Systematics of Streptomycetes from Antarctic Soil

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Declaration

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text of this thesis.

Abstract

Twenty-four soil samples were collected from the Vestfold Hills and Mirror Peninsula in the Antarctic. Fifty-two hyphal actinomycetes were isolated from six of the 24 soils, using micromanipulation. Five of the six actinomycete bearing soils were from sites associated with moss or lichen but no other correlation between the presence of viable actinomycetes and soils characteristics was determined.

Fatty acid and isoprenoid quinone profiles indicated that each of the 52 isolates could be accommodated within the genus *Streptomyces*. Phenotypic characters, fatty acid profiles, and 16S-23Sr RNA intrageneric spacer patterns were compared among all isolates. As a result of these comparisons the 52 isolates were placed into five groups, each group being comprised of strains of a single species. Representative strains were chosen from each of the five groups for further study. Partial 16S rRNA sequence comparison and DNA:DNA hybridization studies indicated that each of the five representative strains was a separate species. These studies also showed that two of the representative strains were known species, *Streptomyces analatus* (DSMZ strain 40361) and *Streptomyces vinaceus* (DSMZ strain 40257). Two other representative strains were not identified as known species, but extensive DNA:DNA hybridization studies are needed to determine their taxonomy accurately within the currently accepted definition of a species. One of the representative strains was identified as a new species.

The diversity of actinomycetes in Antarctic soils is relatively low compared to that in temperate soils. However, the distribution of Antarctic actinomycete strains and their location near moss and lichen beds suggested that these strains were able to survive in the Antarctic and to grow there for at least part of the Antarctic year.

An attempt was made to differentiate between the five representative strains using Amplified Ribosomal DNA Restriction Analysis (ARDRA). However, the ARDRA technique was only effective if differences in 16S rRNA sequences were known so that appropriate enzymes could be selected. Differentiating between strains on the basis of 16S-23Sr RNA intrageneric spacer patterns was a faster and more accurate way of grouping closely related isolates.

Antibiotic compounds were produced by two of the Antarctic strains, indicating the potential of the Antarctic microbiota for biotechnological exploitation.

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Contents

Common Abbreviations Used in This Thesis

DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
PAA	polyacrylamide
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulphate
PCR	polymerase chain reaction
dNTP	deoxy nucleoside triphosphate
UV	ultra-violet
SSC	standard saline citrate
LOI	loss on ignition at 600°C
EC	electro-conductivity
GYM	glucose-yeast-malt
PYI	peptone-yeast-iron
MK	menaquinone
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
RFLP	Restriction Fragment Length Polymorphism
ARDRA	Amplified Ribosomal DNA Restriction Analysis

Chapter 1: Literature Review

1.1 Antarctic Microbiology

Viable bacteria have been isolated from a variety of Antarctic environments, including ice (Abyzov, 1993) snow (Meyer *et al.*, 1967), saline and freshwater lakes (Dobson, *et al.*, 1993; Franzmann, *et al.*, 1988a; Meyer *et al.*, 1967), air (Cameron, *et al.*, 1972; Friedmann, 1982; Meyer *et al.*, 1967), soil (Benoit and Hall, 1970; Boyd, *et al.*, 1970; Cameron *et al.*, 1970; Cameron, 1971; Cameron, *et al.*, 1972; Horowitz, *et al.*, 1972; Meyer *et al.*, 1967; Ramsey and Stannard, 1986) plants (Meyer *et al.*, 1967) and from beneath and within translucent rocks (Friedmann, 1982; Siebert, *et al.*, 1996). Each of these environments has its own characteristics which preclude generalisations about the conditions for life in one being carried over to the others in most instances, and which select for bacteria adapted to their own micro-climates. Since this study pertains to microorganisms isolated from Antarctic soil the following review is restricted to those factors affecting the soil and its microbiota.

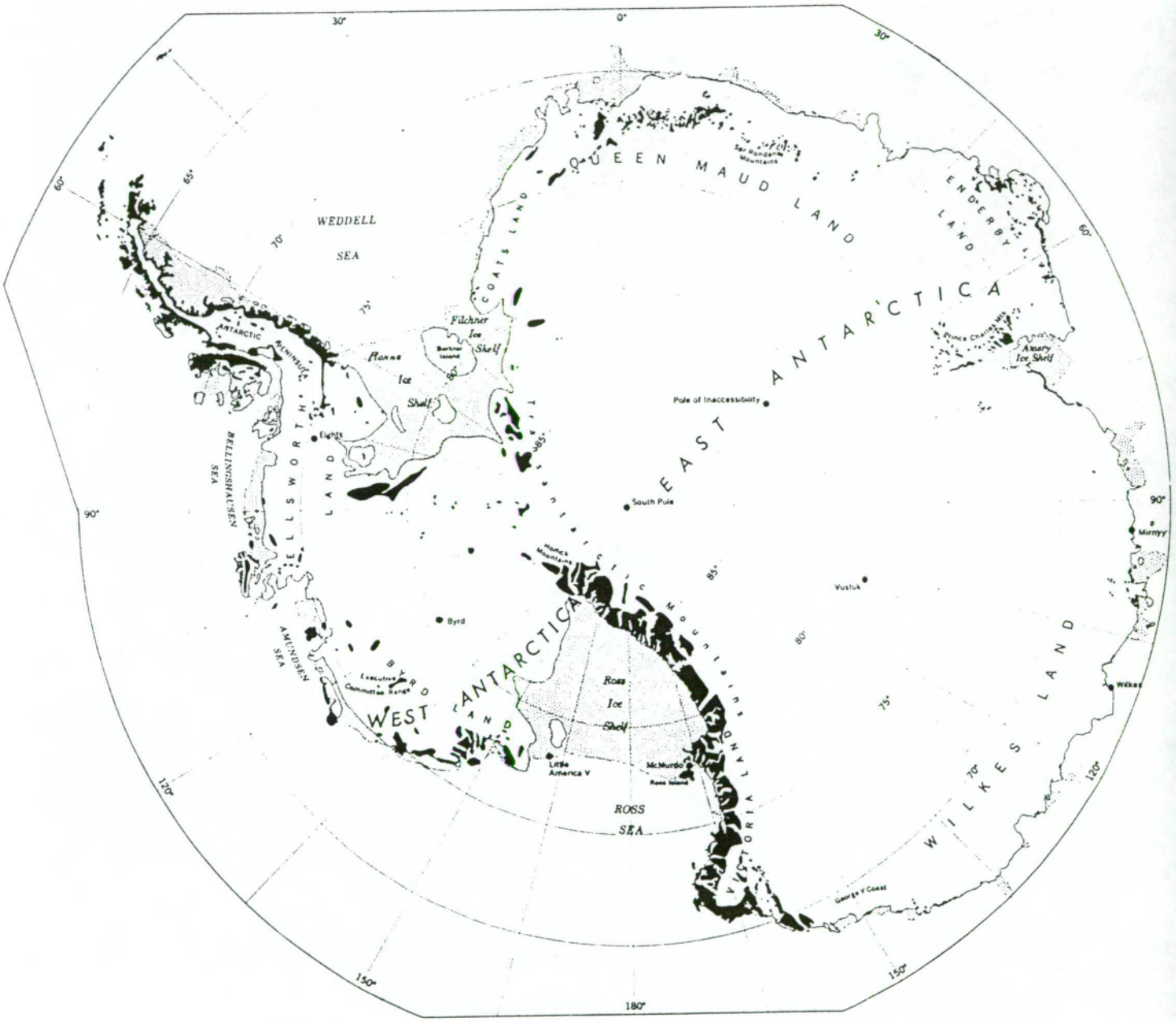
1.1.1 The Antarctic Environment

1.1.1.1 General Climate

The Antarctic climate is one of the least hospitable to life on the Earth's surface. The low temperature, low water availability, lack of light through six months of the year, high levels of ultra-violet radiation during the southern summer, long freeze-short thaw cycle, rapidly alternating freeze thaw cycles during summer, short growth period, hyper-salinity of the lake water, and often of soils, low mineral content of glacial run-off and lack of nutrient input are major obstacles to growth and survival of microorganisms (Benoit and Hall, 1970).

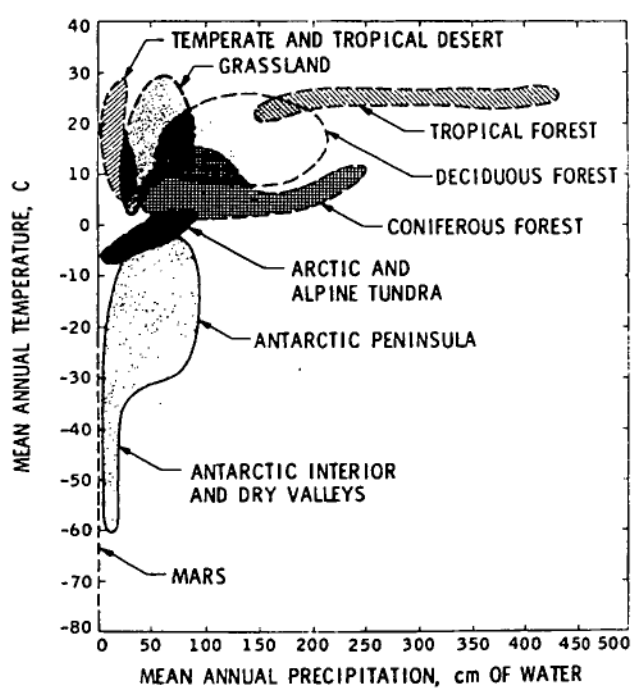
Some areas of the Antarctic continent are so unfavourable to life with regard to water availability and temperature that they have been suggested as test areas for methods of life detection on Mars (Cameron *et al.*, 1976; Horowitz *et al.*, 1972; Meyer *et al.*, 1967) (Fig. 1.2). These two factors are probably the main life limiting factors in the Antarctic environment.

Figure 1.1 The Antarctic Continent. Regions of mountains or exposed rock are shown as dark areas. This map is modified from Fig. 1 Tedrow and Ugolini, (1966).



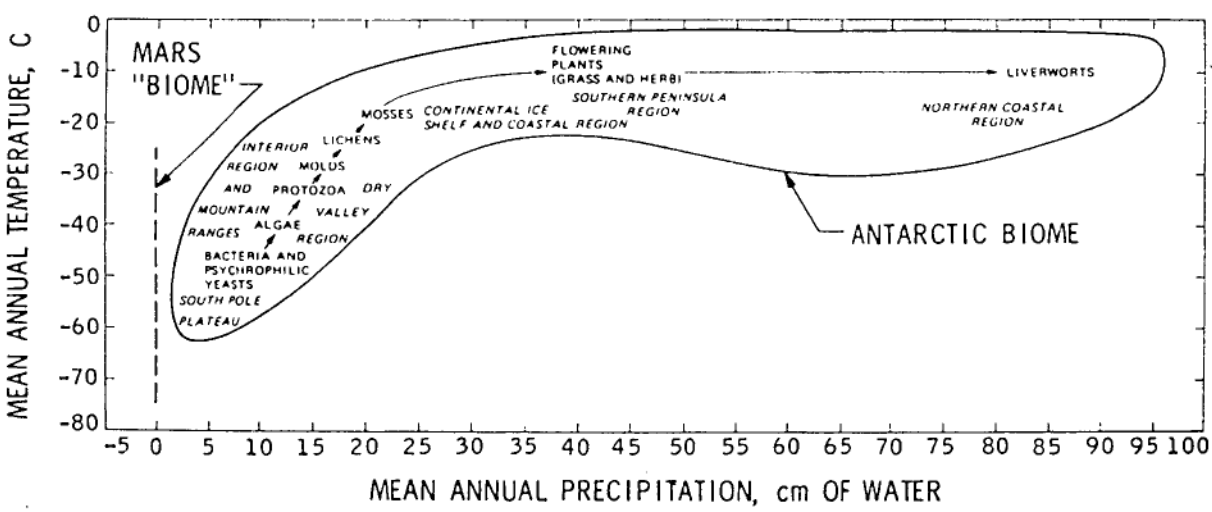
Water vapour content in the air over the Antarctic continent is an order of magnitude less than in temperate climes (Horowitz *et al.*, 1972). Relative humidity in the Dry Valleys recorded a range from 16% to 75% over five summer seasons (1976 - 1981) (Friedmann, 1982). Moisture content of the soil in the Dry Valley was comparable to that of temperate deserts of around 0.3% and in Victoria Land of approximately 1% (Tedrow and Ugolini, 1966). Little snow falls in the Antarctic Dry Valleys, an average of 15g/cm/year (Vishniac and Hempfling, 1979) and the little which does fall is probably sublimated by the strong dry wind, with a relative humidity of 5-10%, which blows into the valley from the interior of the continent (Vishniac and Hempfling, 1979). Some melting snow has been seen in the area and fog which would also provide some moisture (Friedmann, 1982), but these are rare events. Rare though these melts are, they could be important for brief microbial activity (Tedrow and Ugolini, 1966). The relative humidity of the Vestfold Hills and the Mirror Peninsula are higher due to their lower altitude and maritime influences but during the winter, months of sub-zero temperatures mean that water availability is very low and life processes probably come to a stand still.

Figure 1.2: The Antarctic climate compared to other earth climates and that of Mars. From Cameron *et al.*, (1976)



The temperature regime on the Antarctic peninsula is comparable to that of the Arctic and some alpine regions, but the climate of much of the continent, and especially of the interior, is much harsher (Fig. 1.2) (Cameron *et al.*, 1976). Temperatures in the Dry Valleys, for example, range from -15°C to 0°C in the summer and can drop to near -60°C in the winter (Friedmann, 1982). The annual mean temperature varies from -20°C to -25°C. In Victoria Land the temperature of the soil remains below 0°C for 9 months of the year (Tedrow and Ugolini, 1966). However, generalised temperature readings such as these can be deceptive. For instance, (Horowitz, *et al.*, 1972) states that mean air temp for the Dry Valleys is -20°C to -25°C, rising only a little above 0°C in the summer but that the summer ground temperature can rise to 15°C for short periods .

Figure 1.3: The Antarctic biome - Temperature and Annual Precipitation, from: Cameron *et al.*, (1976).



Even in the harsh climate of the Pensacola mountains, soil microhabitats can develop with temperatures well above ambient air and sub surface temperatures (Cameron *et al.*, 1972; Parker *et al.*, 1982). It is difficult to compare temperatures from different areas as air and ground temperatures are usually not reported. Temperatures recorded at Davis Base in the Vestfold Hills were less severe with winter minima recorded at Davis base from 1957 to 1981 varying between -29°C and -40°C (Line 1988) rising to 5°C or more during the summer (Horowitz *et al.*, 1972). The problems caused by low temperatures for microorganisms are low water availability, freeze-thaw damage and a low enzyme activity resulting in a slow rate of growth. A rapid rise and fall in soil surface temperature such as that recorded in the

Dry Valleys (Thompson *et al.*, 1971) creates a serious freeze thaw problem for the microbiota.

1.1.1.2 The Soil

The majority of the Antarctic is covered in ice, in some areas up to several kilometres thick. Soil formation is limited by the scarcity of ice free areas, low temperature, and the low availability of liquid water for weathering (Ugolini, 1970). Ice free rock occurs in two different areas: (1) Regions where dry winds descend from the ice plateau resulting in a low or non-existent snow fall (Friedmann, 1982), and local geography combine in such a manner that the rock is exposed (for example the Dry Valleys); (2) Low lying areas at the margins of the Antarctic where higher latitude, lower altitude and the moderating effect of a maritime climate result in an area free of permanent ice and snow cover. Snow cover may occur in some areas and lie for a number of months especially over winter. Altogether these exposed areas represent only 3% of the Antarctic continent (Bonner and Walton, 1985).

Generally, Antarctic soils are coarse and low in organic content (Ugolini, 1970). They have been formed by physical and chemical weathering of igneous and metamorphic rock (Parker *et al.*, 1982) which is often intense in ice free areas (Tedrow and Ugolini, 1966). In most cases the soil has been formed without appreciable biological input (Tedrow and Ugolini, 1966; Ugolini, 1970) except that found near to the coast and associated with moss and lichen beds or penguin colonies, the latter known as ornithogenic soils (Ugolini, 1972). Antarctic soils often have a high salt content consisting of calcite (calcium carbonate) and gypsum (calcium sulphate) with lesser concentrations of chlorides, nitrates and other sulphates. These high salt areas may be concentrated in patches or dispersed over a wide area depending on local conditions, which is a feature they have in common with hot deserts. The salt comes from seaspray, evaporated seawater, atmospheric aerosols and in some areas has accumulated over millions of years (Cameron *et al.*, 1970). Antarctic soils are usually aerobic except for a few maritime wet moss soils (Vishniac, 1993).

Antarctic Soils can be divided into three broad categories based on climate, organic carbon and moisture availability (Claridge and Campbell, 1985). These are high altitude soils, soils at the continental margins and coastal and island soils.

1. High altitude soils: These soils can be several million years old. They are found in ice free areas of the Trans-Antarctic mountains which extend from northern Victoria Land, across the South Pole to the Pensacola Mountains (see Fig.1.1). These areas have been described as the least favourable for life of all the ice free areas of the Antarctic (Vincent, 1988), though they are not as dry as in the Dry Valleys of southern Victoria Land (Parker, *et al.*, 1982). Two sub types of soils have been described in these areas: Evaporite soils, formed in depressions and low, flat waterways, and having a high salt content; and ahumic soils, that are most common to the dry valleys and are formed by chemical weathering and have a high pH and salt content (Tedrow and Ugolini, 1966; Cameron, *et al.*, 1970). A permafrost layer usually occurs in this soil at depths of 10-30 cm (Tedrow and Ugolini, 1966; Cameron, *et al.*, 1970). The main mechanism of soil formation appears to be chemical weathering (Parker *et al.*, 1982). Nutrient input is very low in these soils, even the overflight of a bird is a rare event (Vishniac and Hempfling, 1979).

2. Soil at Continental Margins: The margins include ice free areas such as the Dry Valleys, Victoria Land, Bunger Hills, Vestfold Hills, Mawson Base, Ross Island, Mirny Base and Enderby Land. These areas are climactically more benign than those at which high altitude soils are found (Vincent, 1988). Some support cyanobacterial mats as well as eukaryotic life, such as moss and lichen, or serve as a brooding area for penguins, all of which contributes an organic component. These organic areas can be quite isolated however, and a moss or lichen bed may exist adjacent to a more exposed area of soil with very little organic content, or a penguin guano enriched soil may lie next to an area only rarely visited by penguins. Chemical weathering again plays a part in the formation of this soil but physical weathering, animal weathering and the deposition of glacial till are also major contributors, their importance varying according to local conditions. Salts may be abundant in some of these soils, especially on the surface. Soil core samples to 313 m taken in the Taylor Valley showed a variation of 0.02% to 0.31% total organic carbon (Vincent, 1988). The soil under the moss and lichen or algal beds may contain 2-3cm of humus over a sand layer which contains traces of organic carbon and which may be up to 10cm deep (Horowitz, *et al.*, 1972). These mosses and lichens contain high concentrations of sugars and sugar alcohols which have the added benefit of acting as compatible solutes, possibly providing some protection for bacteria during freeze-thaw cycles (Ellis-Evans and Wynn-Williams, 1985; Vincent, 1988).

3. Coastal and Island Soils: Relatively high moisture content from sea-spray and frequent drizzle during the summer, marine birds and mammals and a regular freeze thaw cycle all contribute to these relatively highly developed soils. Many parts of the Antarctic peninsula fall into this category, for instance Paradise Harbour where maximum soil and air temperatures of 29°C and 16°C respectively have been recorded. Organic nitrogen content is high in ornithogenic soils because of ammonium release from the guano and to a lesser extent from biomass mineralisation in the plant covered areas. Dense patches of moss, lichen and algae at certain locations can result in peat formation (Vincent, 1988). Three species of vascular plant also occur in some coastal areas of the Antarctic peninsula (Boyd, *et al.,* 1970).

Using the above method of soil classification, the soils of the Vestfold Hills and Mirror Peninsula fall mainly under the classification of soils at the continental margins. The soils of the Vestfold Hills were formed during the last 3000-7000 years by chemical and physical weathering of Archaean gneiss (Line, 1988). Before this time the Vestfold Hills were beneath the sea and so many of the soils are hyper saline and mildly to moderately alkaline. Ice in the soil thaws on average for 58 days per year in the Vestfold Hills but the degree of thawing depends on the salinity of the soil (Line, 1988).

1.1.2 Bacteria in Antarctic Soils

The Antarctic has been a separate continent for approximately 70 million years (Lee, 1993). Organisms bound to the Antarctic continent for this period would have followed a separate evolutionary path from their relatives for this length of time. However, viable wind borne microorganisms (Horowitz, *et al.,* 1972) and viable pollen from non-Antarctic sources rain onto the continent (Liskens, *et al.,* 1992) so the soil of Antarctica is continually re-inoculated. There is debate about the length of time for which the Antarctic continent has had its modern harsh climate. Some estimates being as brief as three million years (Barrett *et al.,* 1992).

A number of psychrophiles isolated from Antarctic environments provide evidence of a unique indigenous microbiota. These psychrophilic and psychrotrophic microorganisms have been isolated from hypersaline lakes in the Vestfold hills (Dobson *et al.,* 1993; Franzmann *et al.,* 1988a; Franzmann *et al.,* 1988b; Franzmann, 1996) and from soil in the Schirmacher oasis (Shivaji *et al.,* 1992). Other psychrophilic and

psychrotolerant bacteria whose taxonomy is uncertain were isolated in earlier studies (Benoit and Hall, 1970; Boyd, *et al.*, 1966; Cameron *et al.*, 1970; Horowitz, *et al.*, 1972).

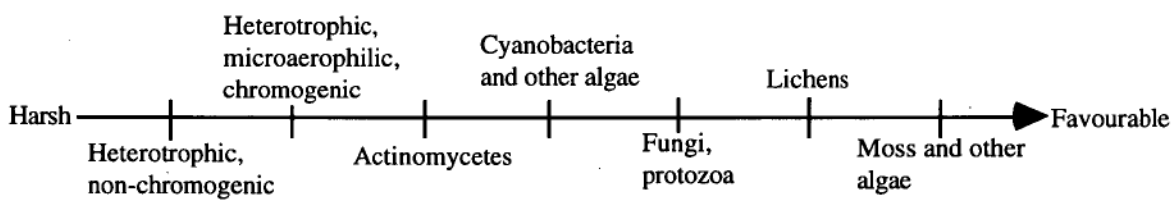
To date, the majority of studies of Antarctic soil microbiota have been along the following lines: A number of soils were collected, the physical characteristics of the soils and the sampling sites were compared, the bacteria counted and identified to genus level and the differences between the soils and bacterial counts, genus diversity, etc. were then discussed. These studies were concerned mainly with the limits of life on Earth and detailed taxonomy of bacterial isolates was not a key issue. In the majority of cases bacteria were only identified to genus or even family level. Most of these studies were also conducted before the advent of molecular taxonomical methods. These new methods have changed our understanding of bacterial phylogeny and taxonomy and have resulted in an altered definition of many taxa previously defined on the basis of morphological and physiological characteristics. Members of the genera *Brevibacterium*, and *Sarcina*, were reported to occur in a number of Antarctic soils but both taxa have recently undergone changes in definition (Vishniac, 1993). Members of the genus "*Achromobacter* " have now been re-accommodated in other genera (Vishniac, 1993). Many bacteria in earlier studies of Antarctic soil may have been assigned to genera which have since been redefined, and if the bacteria were novel, indigenous, or unusual species the chance of inappropriate generic assignment was possibly greater. The methods of isolation used in these studies have varied to such an extent that comparisons between them may be meaningless (Vishniac, 1993). Despite this, it is useful to examine the results from these studies as they give the only information on the distribution and diversity of bacteria in the soil and of the frequency with which bacteria identified as Actinomycetes have been isolated.

Antarctic soil microbiology was first investigated early this century (Ekelof, 1908a and b; McLean, 1919; Pirie, 1912; Tsilinsky, Mlle., 1908). These studies reported that viable bacteria could be isolated from Antarctica but the state of bacterial taxonomy at that time did not allow any detailed investigation of species diversity. Interest was revived in 1960 (Flint and Stout, 1960) and was given impetus by the American space program in the mid sixties when the Antarctic climate was proposed as a model for the investigation of life on Mars (Cameron *et al.*, 1970; Cameron, 1971; Cameron *et al.*, 1972; Cameron *et al.*, 1976; Horowitz *et al.*, 1972). There have also been other

general studies of the soil microbiota since that time (Benoit and Hall, 1970; Boyd *et al.*, 1966; Boyd *et al.*, 1970; Block, 1984; Cameron *et al.*, 1976; Friedmann, 1982; Kerry, 1990; Line, 1988; Meyer *et al.*, 1967; Parker *et al.*, 1982; Ramsey and Stannard, 1986; Shivaji *et al.*, 1992; Vishniac and Hempfling, 1979).

It is possible to describe in relative terms the ability of different groups of microorganisms to survive in the Antarctic soil as conditions for life become more or less favourable (Fig. 1.4).

Figure 1.4 A non-scale representation of the relative chance of survival for different organisms in the Antarctic soil with decreasing harshness of the soil environment. From Cameron *et al.*, 1970



There has been debate about whether some Antarctic soils are capable of supporting any life. Studies of the air and soil in the Dry Valleys (Horowitz, *et al.*, 1972) reported sterile soils and suggested that any microorganisms found in the area were air-borne contaminants. Some of the bacteria isolated from air and soil were identified as species which were also found in temperate climates (Horowitz,*et al.*, 1972). Boyd *et al.*, (1970) reported that microorganisms indigenous to Paradise Harbour would need to be able to grow at a water activity of 0.48 but none of the bacteria isolated from Paradise Harbour soils in this study were capable of doing so. A water activity of 0.6 may be close to the lower limit for microbial life and therefore, as the water activity of the southern Dry Valley soils and Paradise Harbour is less than this, it has been proposed that no microorganism isolated from these site could be considered indigenous (Horowitz, 1979). Greater numbers of viable cells have been reported from around ponds in the Dry

Valleys where the water availability is higher but numbers rapidly decreased, eventually to zero, in samples taken at increasing distances from the ponds (Horowitz, 1979). It was proposed that some of the Antarctic soils in the drier parts of the Dry Valleys are so hostile to life that they are abiotic and that no microorganism is adapted to this harsh environment (Horowitz, 1979). Similar proposals were made for the soils of the Pensacola mountains in which a sparse microbiota had been reported (Cameron and Ford, 1974; Cameron *et al.,* 1972; Friedmann, 1977; Parker, *et al.,* 1982).

Table 1.1: Genera isolated from the soil and air from a range of sites in the Antarctic Cameron *et al.,* (1972).

Genera	Coast of McMurdo Sound		Dry Valleys		Interior		Peninsula	
	Soil	Air	Soil	Air	Soil	Air	Soil	Air
"Achromobacter"	2		4		2			
Arthrobacter	8	2	42	2	4	1	10	2
Bacillus	2	1	12	17	1	5		
Brevibacterium	1	15	2	1				
Corynebacterium	13	2	52	5			3	1
Cytophaga		1						
Flavobacterium	3		1			1		
Micrococcus	5	1	15	7	1	3	2	1
Myococcus			1		1	1		
Nocardia	2		6					
Pseudomonas				1				
Streptomyces	3		7	2	1			

Supporting evidence for the theory of the air borne origin of bacteria in Antarctic soils comes from a study of Antarctic soil and air by Cameron *et al.,* (1972) (Table 1.1). Members of only four of the genera isolated, *"Achromobacter," Nocardia, Flavobacterium* and *Streptomyces,* were found at some sites in the soil but not in the air. Of these four only members of the genera *"Achromobacter,"* and *Nocardia,* were not isolated from the air at any site.

Subsequent studies reported an indigenous yeast from soils in the Dry Valleys, which showed some adaptation to low temperatures and low nutrient availability (Vishniac and Hempfling, 1979). Some evidence has also been presented for an indigenous population in the soils of the Pensacola mountains (Parker, *et al.*, 1982). It has been reported that the recovery of viable cells from Antarctic soils depends to a great extent on the technique used (Vishniac and Hempfling, 1979; Parker, *et al.*, 1982) and in some cases a recovery rate of 100 fold greater numbers of microorganisms can be achieved using an appropriate low nutrient medium as compared to a richer one (Parker *et al.*, 1982). It is possible that some of the soils described as sterile and abiotic (Cameron *et al.*, 1972) may harbour microbiota which are not easily recovered.

The margins of the continent such as the Vestfold Hills and the Antarctic peninsula, have soils which more readily support microbial growth. The presence in these areas of growing moss and lichen beds and cyanobacterial mats (Vincent, 1988; Horowitz, *et al.*, 1972) proves this point. There are also large numbers of microorganisms in the ornithogenic soils, which receive a regular supply of water and nutrients from the penguins. The diversity of bacteria in these marginal soils may not be great. The number of bacteria belonging to different genera isolated from soil using conventional culturing techniques tends to be less in cold climates (Li-Hua *et al.*, 1996) with members of 10-11 different genera commonly isolated from tropical soils compared to four to six from mountains in cool temperate regions.

Some contamination by humans and other animals must be expected in Antarctic soils, especially in the areas near to the Antarctic bases (Vishniac and Hempfling, 1979; Kerry, 1990). There have been reports that isolation of spore forming *Bacillus* sp. and fungi have been increasing since studies were first conducted and were absent from relatively undisturbed areas (Cameron, *et al.*, 1976).

Besides wind and animal borne bacteria, a third possible source of soil microorganisms is meltwater from the ice sheet. Abyzov (1993) isolated a wide range of viable microorganisms from an ice core, including actinomycetes and other eubacteria, filamentous fungi and yeasts. These became less common with depth, but were relatively abundant to a depth of 320m, which was deposited approximately 12 500 years ago.

1.1.2.1 Bacteria from High Altitude Soils

Studies of soils in these areas are not common because of the lack of accessibility. The high altitude soils contain sparse microbiota (Cameron and Ford, 1974; Cameron 1972; Friedmann, 1977), but some viable isolates included algae, aerobic heterotrophic bacteria (mostly gram negative rods) and yeasts (Parker, *et al.*, 1982).

1.1.2.2 Bacteria from Soils at the Continental Margin

The margins include ice free areas such as the Dry Valleys, Victoria Land, Bunger Hills, Vestfold Hills, Mawson base and Enderby Land. Most studies of Antarctic soil microorganisms have been conducted in these areas because they are easier to reach and have the greatest area of exposed soil.

Vestfold Hills and at Mawson Base

Chitinolytic bacteria, fungi and green algae were isolated from soils with low salinity and halotolerant bacteria from soils with high salinity (Line, 1988) in soils of the Vestfold Hills and Mawson Base. The halotolerant isolates are probably indigenous rather than wind borne contaminants.

Pristine environments were reported to be dominated by Gram negative aerobic rods which comprised (68%) of the total isolates, members of the genera *Moraxella Acinetobacter* and *Pseudomonas* were the most common. The remainder were Gram positive pleomorphs (13%), filamentous Gram negative rods with a *Bacillus*-like morphology (12%) and yeasts (7%). Members of the genus *Streptomyces* were the only actinomycetes identified and were dominant in only one sample: an alkaline soil taken from the sea edge. *Streptomyces spp.* were only isolated from two other soils, and all three of the soils in which they were found contained comparatively low microbial numbers.

Nitrogen-fixers were not isolated from any soils but their presence was indicated in 40% of soil through use of an acetylene reduction assay. This suggests that there may be numerous other microorganisms present in Antarctic (and other) soils which are difficult to isolate using traditional methods.

A variety of fungi were isolated from both sites but only members of the genus *Verticillium* were found in pristine soil. In areas of human activity much wider variety of fungi have been isolated than from pristine soils (Kerry, 1990).

Many of the bacteria collected from this area have been lodged in the Australian Collection of Antarctic Microorganisms. In 1995 this collection contained no hyphal actinomycetes (Mancuso, *et al,* 1991).

Dry Valleys

Studies of Antarctic Dry Valley soils have identified *Corynebacterium spp.*, *Arthrobacter spp.*, and *Micrococcus spp.* (Cameron, *et al.*, 1972; Benoit and Hall, 1970), *Planococcus spp.* (Miller and Leschine, 1984), aerobic or microaerophilic heterotrophic mesophiles, *Streptomyces spp.* and *Bacillus spp.* (Cameron, *et al.*, 1970), *Sarcina spp.* ,*Staphylococcus spp.* , *"Achromobacter"spp.*, *Flavobacterium spp.*, *Kurthia spp.*, (Cameron *et al.*, 1976), *Azotobacter spp.* (Benoit and Hall, 1970), filamentous fungi (Vishniac and Hempfling, 1979; Benoit and Hall, 1970; Cameron *et al.*, 1976) yeasts (Vishniac and Hempfling, 1979; Cameron *et al.*, 1976) and algae (Cameron *et al.*, 1976).

Cameron, *et al.*, (1970) divided the microbial population soils in the Dry Valleys into four dominant groups (However, see sections 1.2.1, 1.3.4.1 and Table 1.7 for more recent definitions of Actinomycete taxonomy):

1. Gram positive cocci: *Micrococcus spp.*, and*Myococcus spp.*
2. Soil diphtheroids: *Corynebacterium spp.*, *Brevibacterium, spp.* *Arthrobacter spp.* and related
3. Gram positive and negative rods: *Bacillus spp.* and *Pseudomonas spp.*
4. Actinomycetes, primarily *Streptomyces spp.*

Chromogenic bacteria were the most common in surface samples and non-pigmented, opaque, translucent, or white isolates dominated in sub-surface samples.

The bacteria collected by Cameron (Cameron, 1971; Cameron, *et al.*, 1972) were re-examined by Johnson *et al,* (1978) who identified members of the following genera and their proportion of the total collected: 56% "Coryneform related" including 23% *Corynebacterium,* 20% *Micrococcus,*

7% *Bacillus*, 3% *Nocardia*, 3% *Streptomyces* and 6% *Acinetobacter*, *Flavobacterium* and *Pseudomonas* (Vishniac, 1993).

Benoit and Hall (1970) reported that chromogenic Gram positive cocci dominated in dry soils, whereas in soils near ponds there was a mix of chromogenic Gram positive cocci, Gram negative cocci and rods and some yeasts. They also found that *Micrococcus spp.*, Gram positive spore forming rods, actinomycetes, soil algae and filamentous fungi were in higher numbers in the soils near beds fed by streams of glacial origin.

Greater numbers of isolates were reported at a depth of 15 cm compared to soils at the surface, or at greater depths, when taking samples at sites removed from glacial streams (Benoit and Hall, 1970). This agreed with the findings of Cameron *et al.*, (1970) who found higher numbers of viable bacteria at the icy-permafrost sub-surface layer than in the soil above it. In some of these soils actinomycetes (identified as "primarily *Streptomyces spp.*") were the dominant microflora, but would be absent a few metres away.

Psychrophiles (Benoit and Hall, 1970) made up a greater percentage of the soil population where conditions were favourable for growth (1:1 psychrophilic:mesophilic in favourable conditions, 1:3 in unfavourable). This finding suggests that the psychrophiles may be indigenous, as a more even distribution would be expected if all strains were a result of wind-borne contamination.

Mirny Observatory

In soils near Mirny observatory (Meyer *et al.*, 1967) *Pseudomonas spp.* and chromogenic micrococci were most commonly isolated, with *Corynebacterium spp. Mycobacterium spp.* and *Flavobacterium spp.* also isolated but more restricted in range, and *Streptomyces spp.* and yeasts isolated at many locations but in low numbers. This study also found some filamentous fungi associated exclusively with moss and lichen.

Cape Bird

From Cape Bird the genera *Bacillus spp.*, *"Achromobacter spp."*, *Arthrobacter spp.*, *Aerobacter spp.*, *Pseudomonas spp.*, *Streptomyces spp.*

and a variety of flavobacteria (Greenfield and Wilson, 1981) have been reported.

1.1.2.3 Bacteria from Coastal and Ornithogenic Soils

Investigations of the soil of the Antarctic peninsula have reported much higher bacterial numbers than in soils further south (Boyd, *et al.*, 1970). Numbers were especially high in areas of human disturbance and near the coast. A variety of microorganisms were reported including spore formers, molds, blue-green algae, rotifers, tardigrades and a number of protozoans. Larger eukaryotes included flatworms, colonial algae, lichens, mosses, flies, springtails and angiosperms.

The most common bacteria isolated were identified as *"Achromobacter" spp., Pseudomonas spp.* (Boyd, *et al.*, 1970; Block, 1984) and *Micrococcus spp.* (Boyd, *et al.*, 1970), some chromogenic yellow and orange *Flavobacterium spp.* (Boyd, *et al.*, 1970) and *Alcaligenes spp.* (Block, 1984). Less common were *Bacillus spp.* and yeasts. In the ornithogenic soils *Micrococcus spp.* (Boyd, *et al.*, 1970; Ramsey and Stannard, 1986) *Pseudomonas spp.*, and *Brevibacterium spp.* were common (Boyd, *et al.*, 1970).

The earliest bacterium from Antarctica identified as a streptomycete was taken from a soil of the Antarctic peninsula (Tsiklinsky, 1908).

Bacillus spp., and *Streptomyces spp.* and some fungi were found in some soils from Ross Island (Boyd, *et al.*, 1966). An actinomycete was isolated from Mt Erebus, Ross Island (Greenfield, 1983) which was reported to produce an antibiotic, though a full identification of neither the antibiotic nor the species producing it were provided. Benoit and Hall (1970) also isolated actinomycetes on Ross Island from soils near the Sulfur cones.

1.1.2.4 Actinomycetes in the Endolithic Environment

One of the richest microbial environments in the Antarctic occurs within the cracks of translucent rocks where autotrophic cyanobacteria and eukaryotic algae and support a population of mixed heterotrophs (Friedmann, 1982). Actinomycetes have not been isolated from Antarctic endolithic environments (Siebert, *et al.*, 1996; Friedmann, 1982).

1.1.2.5 Summary: Genera, Sites and Studies and Actinomycetes

Table 1.2: Eubacterial genera isolated from Antarctic soil

Genus reported	Area	Reference
Arthrobacter	Dry valleys	Cameron, *et al.*, 1970; Cameron *et al.*, 1976
	Cape Bird	Greenfield and Wilson, 1981
Micrococcus	Ornithogenic soils,	Ramsey and Stannard, 1986;
	Antarctic Peninsula	Boyd, *et al.*, 1970; Shivaji, 1988
	Dry valleys	Benoit and Hall, 1970; Johnson *et al*, 1978;
		Cameron *et al.*, 1976; Cameron, *et al.*, 1970
Brevibacterium	Ornithogenic soils	Boyd, *et al.*, 1970
	Dry valleys	Cameron *et al.*, 1976; Cameron, *et al.*, 1970
Corynebacterium	Dry valleys	Cameron *et al.*, 1976; Cameron, *et al.*, 1970
	Mirny observatory	Meyer *et al.*, 1967
Mycobacterium	Dry valleys	Cameron *et al.*, 1976; Cameron, 1971
Nocardia	Dry valleys	Johnson *et al*, 1978; Cameron *et al.*, 1976
Streptomyces	Antarctic peninsula	Tsiklinsky, 1908; Boyd, *et al.*, 1966;
	Mawson Base and Vestfold Hills	Line, 1988
	Cape Bird	Greenfield and Wilson, 1981
	Ross Sea area	Benoit and Hall, 1970
	Mirny observatory	Meyer *et al.*, 1967
	Dry valleys	Cameron, *et al.*, 1970; Cameron *et al.*, 1976;
		Johnson *et al*, 1978
Kurthia	Dry valleys	Cameron *et al.*, 1976
Bacillus	Antarctic peninsula	Boyd, *et al.*, 1970
	Cape Bird	Greenfield and Wilson, 1981
	Dry valleys	Johnson *et al*, 1978; Cameron *et al.*, 1976;
		Cameron, *et al.*, 1970
	Ross Sea area	Boyd, *et al.*, 1966
*Staphylocuccus**	Dry valleys	Cameron *et al.*, 1976
"Achromobacter"	Antarctic peninsula	Block, 1984; Boyd, *et al.*, 1970;
	Dry valleys	Cameron *et al.*, 1976
	Cape Bird	Greenfield and Wilson, 1981
Alcaligenes	Antarctic peninsula	Block, 1984,
Sphingobacterium	Schirmacher oasis	Shivaj *et al.*, 1992
Aerobacter	Cape Bird	Greenfield and Wilson, 1981
Azotobacter	Dry valleys	Cameron, 1971

Acinetobacter	Dry valleys	Johnson *et al*, 1978
	Mawson Base and Vestfold Hills	Line, 1988
Myococcus	Dry valleys	Cameron *et al.*, 1976; Cameron, *et al.*, 1970
Sarcina	Dry valleys	Cameron *et al.*, 1976
*Beijerinkia**	Victoria Valley and Ross Island	Ni, 1986
Pleisomonas	Victoria Valley and Ross Island	Ni, 1986
*Xanthomonas**	Victoria Valley and Ross Island	Ni, 1986
*Deinococcus**	Dry valleys	Hirsch, *et al.*, 1985; Siebert and Hirsch, 1988
Nitrobacter	Dry valleys	Cameron, 1971
*Planococcus**	Schirmacher oasis	Shivaj *et al.*, 1988
	Dry valleys	Miller and Leschine , 1984
Moraxella	Mawson Base and Vestfold Hills	Line, 1988
Pseudomonas	Antarctic Peninsula	Block, 1984; Boyd, *et al.*, 1970; Cameron, *et al.*, 1970
	Mawson Base and Vestfold Hills	Line, 1988
	Ornithogenic soils	Boyd, *et al.*, 1970
	Cape Bird	Greenfield and Wilson, 1981
	Dry valleys	Johnson *et al*, 1978; Cameron *et al.*, 1976
	Mirny observatory	Meyer *et al.*, 1967
Flavobacterium	Antarctic Peninsula	Boyd, *et al.*, 1970;
	Cape Bird	Greenfield and Wilson, 1981;
	Mirny observatory	Meyer *et al.*, 1967
	Dry valleys	Johnson *et al*, 1978; Cameron *et al.*, 1976

* These genera are not commonly found in soils of temperate climates (Vishniac, 1993)

There is disagreement in the literature over numbers and proportions of members of different genera present in the Antarctic soil. Cameron *et al.*, (1976) and Johnson *et al*, (1978) identified coryneforms as the dominant microbiota and only a small proportion are identified as members of the genera *Acinetobacter / Pseudomonas / Flavobacterium* compared with Meyer *et al.*, (1967) who identified *Pseudomonas spp.* as the most common isolate. This is probably due to different isolation methods and possibly to different ways of sampling and storage of soil samples.

Most of the strains isolated from Antarctic soils to date are typical of those found in soils of temperate climates (Table 1.2 above). This supports the

theory that there is an indigenous microbiota in the soil, as greater variation in genera would be expected if the majority of isolates were air-borne contaminants.

All actinomycetes identified to species level prior to 1976 were listed by Cameron *et al.*, 1976 (table 1.3). Although known species were identified the state of actinomycete taxonomy up until that time makes it difficult to interpret this data in the light of considerable recent taxonomic revision.

Table 1.3: Actinomycete species isolated from Antarctic soils, from: Cameron *et al.*, (1976).

Mycobacterium sp.	
Myococcus albus [a]	*Streptomyces sp.*
Myococcus ruber [a]	*Nocardia albicans* [a]
Myococcus sp.	*Nocardia flava* [a]
Streptomyces albus [a]	*Arthrobacter spp.*
Streptomyces exfoliatus [b]	*Brevibacterium spp.*
Streptomyces longisporoflavus [a]	*Corynebacterium spp.*
Streptomyces parvus [b]	*Micrococcus spp.*

[a] Possible indigenous species

[b] Possible introduced species

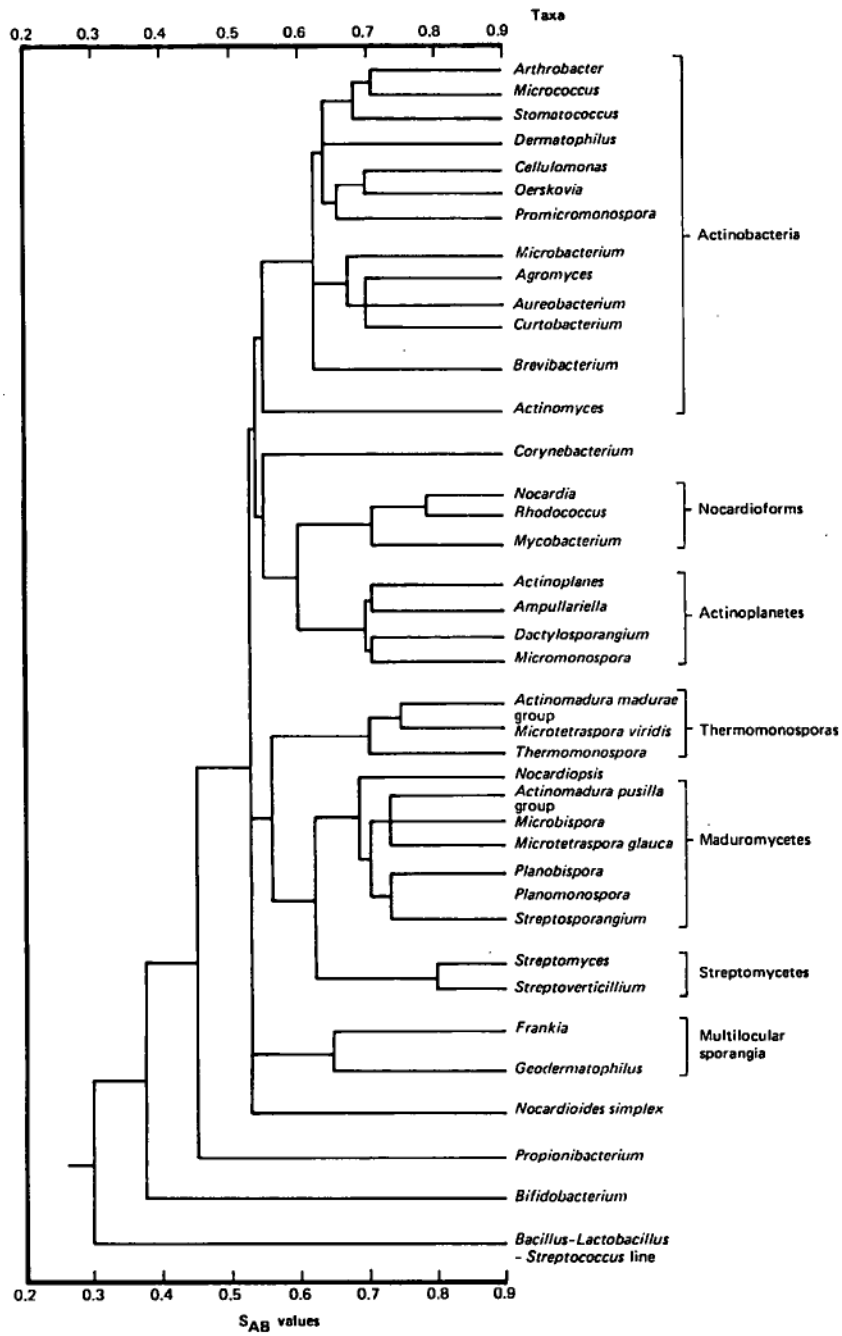
1.2 . Actinomycetes

1.2.1 Description and Biology

The Order *Actinomycetales* can be conveniently divided into two groups for the purpose of this study. These are (i) the fermentative forms which live in mucosal cavities of animals as parasites or commensals (Schaal, 1992) and (ii) the soil or oxidative group which live in other environments, primarily the soil (Lechevalier and Lechevalier, 1985). This division is not related to phylogeny but as this study is concerned with soil bacteria it excludes from detailed consideration most genera in the former group, including *Mycobacterium, Actinomyces, Arachnia, Bacterionema, Bifidobacterium, Arcanobacterium* and *Rothia* (Schaal, 1992). There are some exceptions such as *Actinomyces humiferus* which is a saprophyte (Schaal, 1992).

A traditional description of soil or oxidative *Actinomycetales* is: "Gram-positive bacteria which form branching hyphae at some stage of their development and may produce a spore bearing mycelium" (McCarthy and Williams, 1990). Other characteristics commonly associated with Actinomycetes are that they are mainly found in soil where they play a role in decomposition of organic matter (Lechevalier and Lechevalier, 1985), and that they manufacture enzymes which degrade complex molecules. They are mostly strict saprophytes, aerobic, and widely distributed in nature (Goodfellow and Williams, 1983). This short definition needs clarification and has exceptions. RNA sequencing studies have shown that the possession of branched hyphae (e.g. *Thermoactinomyces*) should not automatically place a bacterium within the order *Actinomycetales,* nor should the ability of the organism to form branching filaments (e.g. *Arthrobacter, Cellulomonas* and *Rothia*) neccesarily exclude it from this taxon (Stackebrandt and Woese, 1981). Members of some of the genera currently placed within this group do not produce spores. Some genera of Gram positive bacteria which are not Actinomycetes do form hyphae, (e.g. members of the genus *Thermoactinomyces*). Indeed, the exact composition and boundaries of the order Actinomycetales and its genera (Buchanan, 1918) have remained open to question and to modification due to continued application of new taxonomic methods (Goodfellow *et al.,* 1988; Williams *et al.,* 1989)."

Figure 1.5: (From Goodfellow 1989) Suprageneric relationships of actinomycetes based on partial sequencing of 16S ribosomal ribonucleic acids.



The use of 16S rRNA sequencing data in the classification of the actinomycetes has resulted in the expansion of this group to include many genera of diverse habit and morphology (Fig. 1.5). Almost any description based on morphology or physiology would have exceptions and actinomycete taxonomy now relies very much on molecular comparisons (Ensign, 1992). The only phenetic characteristics shared by all members of the Actinomycetales is a relatively high level of guanine (G) and cytosine (C) as a percentage of total DNA (>55%) (Srinivasan *et al.*, 1991; Goodfellow, 1989).

This study is focused on actinomycetes which meet three criteria: (i) They must be soil bacteria; (ii) They must have potential for exploitation, i.e. there must be an indication in the literature that the genera in question could produce bioactive secondary metabolites; (iii) They must have a hyphal stage allowing identification and isolation by micromanipulation. There is evidence in the literature that requirements (ii) and (iii) are linked as it appears that microorganisms which form spores are more likely to produce bioactive secondary metabolites (Demain, 1980), and it is usually mycelial actinomycetes which are the spore formers (Labeda, 1990, see Table 1.4). These requirements immediately exclude members of a number of genera including *Corynebacterium* and members of most genera in the Actinobacteria (Fig 1.5) which satisfy neither (ii) nor (iii).

Table 1.4: Antibiotic producing actinomycete genera isolated during the period 1975-1982 and their relative abundance in soil.

Antibiotic producers 1975-82 (Labeda, 1990)	Abundance in Soil
Actinomadura	Rare but possibly underestimated (1)
Actinoplanes	Rare but found in a wide range of soils (1), more common in desert soils and leaf litter (3).
Actinosporangium†	
Actinosynnema	
Ampullariella	
Chainia †	
Dactylosporangium	Rare (1)
Kitasatosporia†	Easily isolated (1)
Microellobospora†	
Microtetraspora	
Nocardia	Quite common (1)
Nocardiopsis	
Pseudonocardia	
Rhodococcus	Some species common (1)
Saccharomonospora	Self heated material (2)
Saccharopolyspora	
Streptoalloteichus	
Streptosporangium	Quite common (1)
Streptoverticillium †	Low numbers but wide range (1)
Streptomyces	Common (1)
*Thermoactinomyces**	
Thermomonospora	Self heated material (2)

References: (1) McCarthy and Williams (1990)
(2) Goodfellow and Williams (1983)
(3) Makkar and Cross (1982)

Genera with a strong hyphal morphology are underlined, (Lechevalier, 1989)

*This genus has now been re-assigned to the *Bacillus-Clostridium-Streptococcus* branch of the Gram-positive bacteria. It is included here because its morphology and secondary metabolite production make it a target in this study despite its taxonomic status. It is interesting to note that members of this genera are spore formers and also producers of antibiotics.

† These genera are now considered to be synonyms of the genus *Streptomyces* (Goodfellow *et al.*, 1986a to d)

The genera in Table 1.4 satisfy the traditional description given above: they are all hyphal at some stage of their development, produce a spore-bearing mycelium, are found in soil, are strictly saprophytic and aerobic.

Hyphal soil actinomycetes often show distinctive morphologies which can aid in identification to generic level. These morphological characteristics include presence or absence of aerial spores, differences in morphology of sporangia when present and number of spores produced, whether spores are motile or not, whether substrate mycelia remain whole or fragment into rods or cocci and any unusual morphological characteristics of the substrate mycelia such as the production of dome-like bodies (*Actinosynnema spp.*) (Lechevalier and Lechevalier, 1985). Unfortunately these morphological characteristics cannot always be relied upon for identification purposes. For example members of the genus *Streptomyces* which are characterised by spore chains morphology, may go bald, or members of the genus *Chainia* (now considered synonymous with *Streptomyces*) that are identified by lipid filled sclerotial bodies may lose the ability to produce them (Lechevalier and Lechevalier, 1985).

Hyphal actinomycetes are typically slow to grow and some may not produce spores for several weeks, though under special conditions one species of *Streptomyces* has been observed to complete its life-cycle within 24 hours of inoculation on a solid medium (Hirsch and McCann-McCormick, 1985).

In liquid culture, hyphal actinomycetes tend to form pellets of various shapes and sizes (Lawton *et al.*, 1989). Morphology in liquid media depends on the species itself, nutrient sources and concentration, inoculum size and pH (Lawton *et al.*, 1989; Glazebrook *et al.*, 1992). Actinomycetes do not usually sporulate in liquid culture but some strains have been reported to do so under certain conditions (Lawton *et al.*, 1989).

Actinomycetes have a single circular chromosome (Hirsch and McCann-McCormick, 1985). Exchange of genetic material is most often by conjugation. They commonly contain extra-chromosomal DNA which has been reported to code for fertility, antibiotic synthesis and resistance, pigment production, exoenzyme production and hyphal differentiation (Hirsch and McCann-McCormick, 1985). This extra chromosomal material may however have a regulatory effect on production of these metabolites rather than coding for them directly as the evidence for plasmid coded antibiotic genes is weak (Aguilar, 1984; Martín *et al.*, 1984). If treated with a

plasmid curing agent, or if frequently sub-cultured a strain can lose all of these characteristics which means these characteristics cannot be reliably employed in numerical taxonomy (Srinivasan *et al.*, 1991). It is important to store isolates in such a way that sub-culturing is not necessary, preferably by freeze drying.

Bacteriophage are commonly isolated from soils in which their actinomycete hosts are abundant (Hirsch and McCann-McCormick, 1985).

1.2.2 Ecology

Actinomycetes can be isolated from a wide range of environments (Kutzner, 1981) including freshwater (although numbers are very low and may be the result of contamination from mud, Labeda and Shearer, 1990), lake sediments, rivers, streams, marine environments (Cross, 1981), salt marshs (Hirsch and McCann-McCormick, 1985), fodder and related materials (Hirsch and McCann-McCormick, 1985) and air (Lloyd, 1969). Their primary niche however is soil (Hirsch and McCann-McCormick, 1985) from which members of over 20 genera having been isolated (Hirsch and Christtensen, 1983).

Actinomycetes can be recovered from most soils in relatively high numbers where they make up 1-20% of the total population (excluding members of the genus *Arthrobacter*) of bacteria (Hirsch and McCann-McCormick, 1985). Typically 10^4-10^7 colony forming units/g of dry soil are recovered (Hirsch and McCann-McCormick, 1985) although this may not give an accurate picture of proportions of active bacteria in the soil because most of the colonies are probably isolated from spores (Williams, 1978). Studies based on direct recovery of plasmid DNA from soil report that viable counts can underestimate spore and mycelial propagule numbers by a factor of greater than 100 (Wellington *et al.*, 1992).

The most numerous bacteria in soil are members of the genera *Arthrobacter, Streptomyces,* the pseudomonads and sporulating bacilli (Hirsch and McCann-McCormick, 1985). Other commonly found non-Actinomycete genera include members of the genera *Clostridium, Azotobacter,* and *Lactobacillus.*

Streptomyces spp. are ubiquitous in soil and are the most numerous of the Actinomycetes (Goodfellow and Williams, 1983) after *Arthrobacter.* The

next most common actinomycetes are, in descending order, the members of the genera *Micromonospora,* (up to 10^4 - 10^5 colony forming units/g of dry soil, Labeda and Shearer, 1990) *Actinoplanes, Actinomadura* and *Nocardia* (Lechevalier and Lechevalier, 1985).

The proportions of members of these genera in the soil can change depending on the condition of the soil. Water content and the proportion of organic matter are the two most important factors (Kutzner, 1981). Many actinomycetes are obligate aerobes and prefer moderate levels of moisture rather than waterlogged soil (Williams *et al.,* 1972). Very dry conditions also inhibit their growth but although total numbers may decrease, actinomycetes can increase their proportion within the microbial population in a drought, from 1-20% up to 70% or even 98% in one isolated case (Hirsch and McCann-McCormick, 1985). This increase of proportion is possibly due to the desiccation resistance of actinomycete spores and vegetative mycelia which contain a high level of trehalose (Ensign, 1992) a sugar associated with tolerance to osmotic stress. Actinomycetes are however less drought tolerant than fungi (Hirsch and McCann-McCormick, 1985).

If actinomycete spores are desiccated or stored in an area of low relative humidity they can remain viable for a number of years, the exact length of time for which they can survive is uncertain (Hirsch and McCann-McCormick, 1985). In environments with a high relative humidity there is a rapid loss of spore viability (Hirsch and McCann-McCormick, 1985). The spores are only slightly more resistant to heat than vegetative mycelia and it is thought that their function is more for dispersion than long term survival (Hirsch and McCann-McCormick, 1985).

High levels of complex organic matter favour the presence of actinomycetes. They appear quite late in the colonisation of fresh organic material (Hirsch and McCann-McCormick, 1985) after the simpler nutrients have been mostly consumed. They can produce a range of enzymes which are capable of degrading the more resistant high molecular weight polymers such as chitin, starch, pectin, certain hemicelluloses, and lignin (Hirsch and McCann-McCormick, 1985).

Actinomycetes are usually intolerant of acidity but can be divided into two groups on the basis of their pH growth optima. The majority are neutrophiles preferring a pH range of 5 to 9 with an optimum at pH 7. The

minority are acidophiles with a pH range of 3.5 to 6.5 and an optimum at about pH 5. There are a few species capable of growth above pH 9 (Hirsch and McCann-McCormick, 1985).

Most actinomycetes are mesophilic. Thermophilic strains are isolated less often. There have been no reports of psychrophilic actinomycetes to date (Hirsch and McCann-McCormick, 1985).

It has been suggested (Hirsch and McCann-McCormick, 1985) that there is some advantage for actinomycetes in living in the plant rhizosphere as their numbers appear to be highest near the surface, numbers dropping steadily down to a depth of 10-15cm. A positive correlation between actinomycete and vegetation diversity (Xu *et al.*, 1996) has been demonstrated which supports this theory.

1.2.3 Biotechnological and Biomedical Potential of Actinomycetes

1.2.3.1 Bioactivity of Actinomycetes

Actinomycetes are responsible for production of two thirds of the 6000 known antibiotics (Bull *et al.*, 1992). It has been suggested that the rate limiting step in the discovery of new bioactive molecules is the isolation and screening of microorganisms (Goodfellow and O'Donnel 1989). Gottlieb (1976) reported that detection of anti-microbial compounds may depend on the variety of test organisms used for screening, with a wide range of test organisms giving the best results.

Actinomycetes do not only produce antibiotics. They also produce many enzymes and metabolites with wide ranging applications (table 1.5) and it has been claimed that they are the most widely biotechnologically exploited group of bacteria (Labeda and Shearer, 1990). By 1984 there were 4607 patents on products and processes directly attributable to actinomycetes (Labeda and Shearer, 1990). Antibiotics and other non-enzymatic bioactive compounds produced by actinomycetes are often referred to as secondary metabolites. Secondary metabolites have been defined as "A naturally produced substance which does not play an explicit role in the internal economy of the organism that produces it" (Maplestone *et al.*, 1992).

There are large numbers of secondary metabolites of identified chemical structure but unidentified physiological activity. In the past it has been

suggested that these secondary metabolites were inactive by-products. As with antibiotics, wider screening programs have identified a bioactive function for many compounds previously thought to be inert (Maplestone *et al.*, 1992) and it is now more commonly assumed that no secondary metabolite, or very few of them, is biologically inactive (Vining, 1992).

Table 1.5: Activity of some compounds with biotechnological applications produced by actinomycetes other than anti-bacterials.

Activity	Genera	Reference
Anti-eukaryotics		
Anti-helmetic, insecticidal	*Streptomyces*	Labeda and Shearer, 1990
Anti-parasitic	*Actinomadura*	Labeda and Shearer, 1990
Anti-viral	*Streptoalloteichus*	Labeda and Shearer, 1990
Anti-fungal	*Actinomadura*	Bull *et al.*, 1992
Enzyme inhibitors		
Reverse transcriptase inhibitor	*Excellospora*	Labeda and Shearer, 1990
Platelet activating factor antagonist	*Streptomyces*	Labeda and Shearer, 1990
Protein Kinase C inhibitor (anti-	*Nocardiopsis*	Labeda and Shearer, 1990
tumour activity)	*Streptomyces*	Nishizuka, 1986
Angiotensin - 1 converting enzyme	*Micromonospora,*	Labeda and Shearer, 1990
inhibitor (anti-hypertensive)	*Actinomadura,*	Nakatsukasa *et al.*,1985
	Streptomyces	
Anti-tumour agent	*Saccharothrix*	Labeda and Shearer, 1990
Enkephalinase B inhibitors	*Kitasatosporia*	Labeda and Shearer, 1990
Adenosine deaminase inhibitor	*Actinomadura*	Labeda and Shearer, 1990
5'-nucleotidase inhibitor	*Nocardioides*	Labeda and Shearer, 1990
Immunosupressant	*Actinomadura*	Labeda and Shearer, 1990
Enzymes		
Transferases	*Streptomyces*	Hartung, 1989
Hydrolases	*Streptomyces*	Hartung, 1989
Restriction enzymes	*Streptomyces*	Hartung, 1989
Isomerases	*Streptomyces*	Hartung, 1989 and Srinivasan *et al.*, 1991
Oxido-reductases	*Streptomyces*	Hartung, 1989 and Srinivasan *et al.*, 1991
Hormone-like activity		
Immunomodulator	*Kitasatosporia*	Labeda and Shearer, 1990
Growth promoter in ruminants	*Kibdelosporangium*	Labeda and Shearer, 1990

Members of the genus *Streptomyces* are responsible for the majority of these compounds and processes. Over half the antibiotics discovered between 1945-1978 were from *Streptomyces spp.* (Williams and Vickers, 1986). Members of this genus are the most common and easily isolated of antibiotic producing actinomycete genera which may account for their dominance in secondary metabolite discovery. In recent times, more antibiotics and other bioactive compounds have been isolated from members of other actinomycete genera as in recent years these were selectively targeted (Labeda and Shearer, 1990; and see section 1.2.4 below). Members of the genera *Micromonospora* and *Actinoplanes* have become especially prominent in this respect which is not unexpected given that they are the next most commonly isolated groups from soil after *Streptomyces spp.* (Lechevalier and Lechevalier, 1985).

Particular families of antibiotic compounds sometimes seem to be produced by members of particular genera, for example aminoglycoside, macrolide and ansamacrolide antibiotics are produced by members of genus *Micromonospora* and depsipepetides by members of genus *Actinoplanes* (Labeda and Shearer, 1990) but this apparent group specificity may be an artefact of screening protocols. It is possible that these group specificities are a result of screening for a particular class of antibiotic in a particular genera once the two become associated. A large number of structurally unrelated compounds are produced by members of the genus *Streptomyces.* Conversely, compounds belonging to the same family of related structures are produced across a number of actinomycete genera (Crandall and Hamill, 1986). This indicates that families of compounds are not necessarily genera specific although the production of a specific structure is usually strain specific (Srinivasan *et al.*, 1991; Vining, 1992). A single strain of actinomycete can produce more than one antibiotic (Srinivasan *et al.*, 1991; Champness *et al.*, 1992; Berwick, 1988). It could therefore be useful to screen strains belonging to genera for compounds showing activities for which they have not been previously screened (Labeda and Shearer, 1990).

Production by members of different genera of related compounds may occur for one of a number of reasons, including an ancestral origin, gene transfer or multiple origins and convergent evolution (Embley and Stackebrandt, 1994). The transfer of antibiotic resistance genes from actinomycetes to other bacteria also suggests a transfer of antibiotic production genes, which are often closely linked to resistance genes, is likely (Kirby, 1992).

As well as producing useful compounds, actinomycetes have been reported to have some applications as whole organisms for nitrogen fixation and gold accumulation (Srinivasan *et al.*, 1991).

1.2.3.2 Role of Secondary Metabolites in Actinomycetes

The role of many secondary metabolites in the environment is unknown. It has been argued that these compounds confer a selective advantage on the producer by interacting with other organisms in such a way as to enhance the producer's chance of survival (Maplestone *et al.*, 1992). The biosynthetic pathways involved in secondary metabolite production, and regulation of their production, are often more complex than those for primary metabolites and therefore represent a significant proportion of the bacterium's metabolism. This supports the theory that secondary metabolites confer a selective advantage, else the waste of energy in producing them is inexplicable (Maplestone *et al.*, 1992; Vining, 1992). This selective advantage may be conferred by the action of secondary metabolites in either of two ways: i) by action on competing microorganisms, ii) by acting on the producing species as a hormone-like regulator of growth, reproduction and differentiation (Vining, 1992). It is likely, given the wide range of structures and functions known that different compounds fulfil both these roles through a variety of mechanisms (Vining, 1992).

Secondary metabolites production is not limited to the actinomycetes. They are also produced by other bacteria, algae, coral, coelenterates, plants and "lower" animals (Maplestone *et al.*, 1992). Maplestone *et al.*, speculate that secondary metabolites are produced by those organisms which lack an internal immune system, playing a role in defence against pathogens, predators and competitors. The evidence that actinomycetes produce antibiotics in the environment is not conclusive - they can be induced to produce antibiotics in soil under controlled conditions (Gottlieb, 1976; Demain, 1980) but antibiotics have not been isolated from environmental soil samples (Maplestone *et al.*, 1992; Williams and Vickers, 1986). Indirect evidence that antibiotic producing strains compete better in the natural environment than non-producing strains of the same species lends support to the argument that they do play this role in nature (Howell and Stipanovic, 1983). The linkage of antibiotic resistance and antibiotic production genes (Hopwood, 1988) suggests that these compounds have undergone complex evolution. The presence of the antibiotic resistance genes in soil microorganisms which do not produce antibiotics themselves

is further evidence for the role of antibiotics in inter-species competition (Maplestone *et al.*, 1992).

Some secondary metabolites appear to have a role in auto-regulation in actinomycetes. The control of cellular differentiation in certain hyphal species by some compounds has been reported (Beppu, 1992).

It has been suggested that antibiotics have some role in the production of spores (Demain, 1980). The evidence for this is that: almost all spore producing microorganisms also produce antibiotics; antibiotic concentrations at sporulation tend to be inhibitory to vegetative growth of producers; polypeptide antibiotics are produced just prior to and during sporulation in bacilli; depletion of particular nutrients will induce both sporulation and antibiotic production; sporulation and antibiotic production genes tend to cluster on the genome (Demain, 1980). However, some mutants have been produced which are capable of sporulation without antibiotic production - why then this close link? Demain (1980) suggested that there are evolutionary advantages in having the two occur simultaneously, for example, the antibiotic may be included in the spore itself to act as an inhibitor of sporulation until conditions are right. Antibiotics have been found which do inhibit sporulation in *Streptomyces spp.* and antibiotics are found in the spores of particular actinomycetes (Demain, 1980).

Secondary metabolite production is considered a result of modification of the metabolic pathways of primary metabolism in ancestral organisms which produced a compound giving some advantage. Subsequent combinations and modifications of such pathways led to the large diversity seen today (Vining, 1992).

1.2.3.3 Control of Secondary Metabolite Production

Antibiotic production in actinomycetes usually occurs at stationary phase (Srinivasan *et al.*, 1991) although this is not always easy to determine in filamentous organisms and may be confused by the formation of pellets in liquid media where different areas of the pellet, i.e. interior and exterior, may be at different stages of growth (Doull and Vining, 1990).

High antibiotic production relies on optimal concentrations of nutrients, pH, viscosity, levels of inorganic phosphorous, metal ions and oxygen,

organic nitrogen sources and metabolic carbon levels and temperature (Srinivasan *et al.*, 1991). Most of these optimal conditions are strain specific and must be determined on a case by case basis (Srinivasan *et al.*, 1991). Conditions which favour antibiotic production are not necessarily those which favour rapid and prolific growth. Slow growth and low levels of certain nutrients tend to stimulate greater production (Srinivasan *et al.*, 1991). The mechanisms which control antibiotic production are also applicable to other secondary metabolites (Martin and Demain, 1980). Genetic regulation of antibiotic production has been studied in detail in *Streptomyces coelicolor* (Champness *et al.*, 1992) where controls were reported to operate at the level of transcription. Other studies have been done on the regulation of Streptomycin biosynthesis in *Streptomyces griseus* and *Streptomyces glaucescens* (Distler *et al.*, 1992). These studies reported very complex regulatory mechanisms and showed some differences between the two species.

It is possible to maximise antibiotic production by varying growth conditions, but when screening actinomycetes for production of secondary metabolites, it is difficult to know what conditions to apply, except that media rich in simple nutrients should not be used. Originally the media used to screen for antibiotic production provided carbon and nitrogen in complex forms such as D-glucose for carbon and Soya bean meal for nitrogen (Rake and Donovik, 1946). More recently synthetic or semisynthetic media were used for the same purpose (Berwick, 1988).

1.2.4 Isolation of Novel Actinomycetes

The importance of actinomycetes as producers of bioactive secondary metabolites has resulted in a large number of studies devoted to methods for isolation of new strains. It generally appears that the more distantly related organisms are, the less probable it is that they will produce the same secondary metabolites (Lancini and Parenti, 1982). There is value in developing new methods and exploring new niches in order to exploit a wider range of organisms. The methods used vary according to the sample source and members of which genus is targeted for isolation. A problem common to all methods is that it is impossible to know the best conditions for isolation of new strains.

One or more of three methods are commonly used when trying to isolate novel microorganisms belonging to specific genera or families (Lechevalier and Lechevalier, 1985):

1. Sampling of sites favourable for the target organism.
2. Pretreatment of samples to remove competitor microorganisms.
3. Selective media which favour the growth of the target microorganisms.

1.2.4.1 Selection of Sampling Sites

As discussed in section 1.2.2 above, actinomycetes occur in a wide range of habitats, but most commonly in soil. The problem with isolating actinomycetes from the soil is not that they are difficult to find but rather that the more common soil bacteria, including members of actinomycete genera such as *Streptomyces* and *Arthrobacter,* overwhelm other less common or slower growing strains during isolation.

Streptomycetes are responsible for the great majority of the antibiotics which originate with actinomycetes (Lechevalier and Lechevalier, 1985) but many of the more recently discovered compounds originate with members of non-streptomycete genera (Lechevalier and Lechevalier, 1985). This is probably because the commercial interest in actinomycetes has resulted in the development of methods which target non-streptomycete actinomycetes. It can be more efficient to target these "rare" actinomycetes, which are less commonly isolated, and therefore more likely to produce novel compounds, than to screen many streptomycetes, a large proportion of which will probably produce previously patented antibiotics.

The soil is therefore still a good place to look for new strains but often soils from unexplored environments are targeted or pretreatments and selective media are used to remove streptomycetes and encourage members of other species of actinomycetes to grow (Williams and Wellington, 1982b). Non-soil environments have also been targeted with some success (Jiang and Xu, 1996; Takizawa *et al.,* 1993).

1.2.4.2 Pretreatment of Samples

There are a number of pretreatments to which environmental samples are routinely subjected when looking for actinomycetes. These include: air drying; drying-wetting; membrane filtration; heating; chemical; baiting;

positive chemotaxis (Labeda and Shearer, 1990); phage depletion of members of unwanted genera (Kurtböke *et al.*, 1992a and b). Combinations of these techniques are often employed (Labeda and Shearer, 1990; Williams and Wellington 1982a and b; Goodfellow and Williams, 1986).

A Note on Sporulation

As many actinomycetes produce spores that may exist in greater numbers than vegetative hyphal cells in the soil it is important to provide conditions favourable for sporulation during isolation. A study by Hirsch and Ensign (1976a) reported that *Streptomyces viriochromogenes* spores would germinate most readily at neutral or slightly alkaline pH at 35°C and required the presence of CO_2 . Spores also tended to germinate less readily ten days after production. The authors noted that these conditions were only known for one strain, *Streptomyces viriochromogenes,* and that this strain is a poor producer of antibiotics. Some antibiotic producing strains have spores which are more difficult to germinate. In further studies Hirsch and Ensign, (1976b) reported that *Streptomyces viriochromogenes* spores could be induced to germinate more readily if they were first heat treated. Maximum rate of germination at 60°C was achieved after 1 minute. A longer period at this temperature caused a rapid decrease in the germination rate of spores. Equivalent rates of germination were achieved by exposure to lower temperatures for longer times (5 minutes at 55°C, 20 minutes at 50°C and 50 minutes at 45°C) and it was noted that increasing the time of exposure at these lower temperatures did not decrease rates of germination. Spores older than four weeks would not germinate spontaneously and could only be induced to do so by heat activation.

Drying

Drying of soil and leaf litter samples for periods of 7 days at 28°C was reported as an effective method of recovering actinoplanetes from such samples (Makkar and Cross, 1982). This treatment kills most Gram negative bacteria in the sample. The Actinoplanetes sporangium survives the desiccation and releases motile spores when re-hydrated.

Heat

Pre-treatment with heat, either moist or dry, is a common technique in targeting actinomycetes. A mild moist heat pre-treatment, 45°C for 2 hours

or 50°C for 10 minutes has been reported to eliminate non-actinomycetes from samples. More extreme temperatures, such as 120°C dry heat for 1 hour have been used to isolate members of some genera with more resistant spores such as *Micromonospora, Microbispora , Microtetraspora* and *Streptosporangium* (Labeda and Shearer, 1990; Hayakawa *et al.,* 1996).

Membrane Filter

This technique makes use of the hyphal growth of actinomycetes which tends to penetrate more deeply than other bacteria into agar media. A cellulose-ester membrane filter is laid over a medium favourable to actinomycete growth and inoculated with the sample. The plate is incubated until growth of bacteria is observed. The filter is then removed and the plate re-incubated until actinomycete colonies appear (Hirsch and Christensen, 1983).

Filters have also been used to isolate actinomycetes from water, by filtering the water sample, then placing the filter, collecting side down, on media and incubating until actinomycete colonies are observed (Trolldenier, 1967).

Chemical

Chemical pretreatment with phenol has been reported to eliminate non-actinomycetes from sediments (Pisano *et al.,* 1986) and other samples (Labeda and Shearer, 1990). The phenol concentration was diluted prior to plate inoculation in order to dilute its toxic effect. Other chemical pretreatments include the use of quaternary ammonium compounds, (members of genera *Mycobacterium* and *Rhodococcus*) and sodium hypochlorite to isolate members of the genus *Frankia* (Labeda and Shearer, 1990).

Bacteriophage

Pretreatment of soils with strain specific phage was reported as an effective method for reducing unwanted organisms from samples without the risk of removing actinomycetes which might be sensitive to other treatments such as heat, drying and antibiotics. This method was reported to reduce numbers of *Streptomyces spp.* (Kurtböke *et al.,* 1992a; Long and Amphlett, 1996) and non-actinomycete thermophilic bacteria, including *Bacillus spp.* and *Pseudomonas spp.* (Kurtböke *et al.,* 1992b) in soil prior to inoculation.

It was reported to increase the yield of the desired actinomycetes in both studies (Kurtböke *et al.*, 1992a; Kurtböke *et al.*, 1992b) and also allowed strains to be isolated which belonged to genera which were not found in the control isolations which used conventional methods.

Baiting

Actinoplanetes can be recovered by placing soil samples in water and floating pollen or hair on the surface. The floating particles are then colonised by motile zoospores from actinoplanetes and can be removed for further treatment (Makker and Cross, 1982). This technique is usually used in combination with other methods such as air drying and the use of selective media. For example, Orchard and Goodfellow, (1977) used paraffin wax-covered rods as bait for isolation of *Nocardia,* again in combination with selective media. This method was considered easier and faster than the other methods in use at the time. The majority of strains recovered were members of the genus *Actinoplanes,* but there were also some members of the genera *Ampullariella, Amorphosporangium* and *Spirillospora.*

Palleroni (1980) employed a different baiting technique to isolate Actinoplanetes. A capillary containing a solution of KCl was pushed into a soil sample. Actinoplanete zoospores which are chemotactic towards KCl entered the capillary, which was then removed, and the contents expelled onto growth media. Mertz (1980) used sterile blades of grass floating on water as a bait to isolate two novel species in the genus *Planomonospora.*

1.2.4.3 Selective Media

Selective media are those which favour the growth of the desired microorganism over all others and those which contain antimicrobials effective against other microorganisms but not against those microorganisms which are desired. Ideally a selective medium should allow rapid growth of the target organism while inhibiting that of all others. In practice this is rarely possible and almost never so if the target organism is a new species which must be allowed a broad range of possible growth parameters.

Development of such selective media was originally through trial and error (Vickers *et al.*, 1984). More recently the use of databases containing detailed

physiological information on antibiotic producing actinomycetes have been utilized.

Vickers *et al.*, (1984) used the physiological data from Williams *et al.*, (1983a) to make media which would select against the large *Streptomyces albidoflavus* cluster which dominated their soil samples and for other actinomycetes in general. They reported success in decreasing proportions *St. albidoflavus* but also concluded that it was not possible to devise one medium which would select for streptomycetes. Rather, a range of selective media should be used, a principle that can be extended to the actinomycetes as a whole.

Huck *et al.*, (1991) constructed a database by characterizing 74 soil bacteria against 43 physiological tests and comparing the results of this characterisation to their ability to produce antibiotics. Their purpose was to determine which of the 43 substrates and conditions favoured the growth of antibiotic producers over other bacteria and to use these substrates and conditions to produce new selective media. The most favourable media were those which had proline and humic acid as sole carbon and nitrogen sources, or asparagine as a sole nitrogen source and which contained certain vitamins. The media which were developed were reported to increase the percentage of actinomycetes compared with total isolate numbers and the percentage of isolates producing antibiotics.

Generally, media for selective isolation of actinomycetes tends to be nutrient poor. The nutrients used are usually complex because actinomycetes can utilize such substrates more readily than many other common soil bacteria (Labeda and Shearer, 1990).

Chitin agar was reported as a particularly good medium for isolation of bioactive actinomycetes (Pisano *et al.*, 1992). A high proportion of chitinolytic actinomycetes isolated using this medium showed antimicrobial activity. The correlation between chitinolytic activity and production of bioactive compounds was so good that the authors suggested there might be a connection between the two, and that chitinolysis might be used as an indicator of bioactivity.

A wide range of antibiotic compounds have been used to selectively isolate members of different genera of actinomycetes. Antibiotics specific for fungi

such as cyclohexamide and nystatin are routinely used in the isolation of actinomycetes (Labeda and Shearer, 1990).

1.2.4.4 Other Methods of Isolation

Visual

Bacteria with distinctive morphologies such as the hyphal actinomycetes are particularly suited to isolation by physical methods that rely on direct observation where this is possible.

The Skerman micromanipulator (Skerman, 1968) has been used for this purpose and appears to be a useful and rapid method for the isolation of hyphae from particular environments, particularly liquid ones where actinomycetes have grown into hyphal pellets that are easily recognised (Blackall, 1991; Blackall *et al.*, 1989).

Another visual method uses a non-destructive laser beam to hold onto a single bacterial cell in a suspension and separate it from other cells. This method has been reported to allow axenic isolation of *Arthrobacter* sp. from rumen fluid, which could not be previously isolated through conventional methods (Mitchell *et al.*, 1992). This method could be useful for the isolation of bacteria with known morphologies from mixed cultures where they are few in number or poor competitors or both which are unlikely to be isolated using more conventional techniques.

Molecular Applications

One important aspect of screening is trying to avoid testing the same organism repeatedly. A combination of molecular and established methods has been suggested as the most efficient way of identifying bacteria efficiently and so avoiding this problem (Stackebrandt *et al.*, 1991).

Combinations of methods

It is usual when targeting any group of actinomycetes to use a combination of methods and media rather than relying on one alone (Jiang and Xu, 1996; Makker and Cross, 1982; Orchard and Goodfellow, 1977; Takizawa *et al.*, 1993; Vickers *et al.*, 1984). This is especially true when looking for novel species with unknown preferred growth conditions.

1.3 Taxonomy

1.3.1 Introduction to Taxonomy

The earliest attempt at a phylogenetic classification of bacteria which included the actinomycetes dates back 60 years (Kluyver and van Niel, 1936). These early studies based phylogenetic classification on morphological characteristics which led to the hyphal forms of the actinomycetes being placed in a class on their own. Actinomycetes, due to their complex morphology, were assumed to have evolved later than supposedly simpler forms, the simplest and therefore most primitive being the cocci (Embley and Stackebrandt, 1994). Most systems of classification made no attempt to base their system of classification on phylogenetic relationships, and morphology and pigmentation were heavily relied upon (Goodfellow *et al.*, 1992; Williams *et al.*, 1984). Actinomycete genera were usually described on the basis of the shape of their sporing bodies and substrate mycelia. These characters were chosen because they were the most easily defined which could be used at the time. This approach was acknowledged by those who used it to be useful for identification but arbitrary and not based on the phylogenetic relationships between different species (Williams *et al.*, 1983a). As the science developed biochemical and physiological characters were incorporated in the development of actinomycete taxonomy but there was no standardisation of methods (Goodfellow *et al.*, 1992).

Actinomycetes were sought after as producers of novel secondary metabolites. Journals and authors who published new antibiotic structures were less concerned with standardisation of bacterial taxonomy than with good chemistry, and the majority of the 3000 *Streptomyces* species named by 1970 had been described inadequately in the patent literature (Williams *et al.*, 1983a; Trejo, 1970).

Many different systems of classification were devised by bacteriologists using combinations of a wide range of different characteristics, resulting in a confusing array of conflicting results (Williams, *et al.*, 1983a). In 1964 the International Streptomyces Project was established in an attempt to overcome the taxonomic confusion associated with this large and important genus. The project attempted to describe all strains of the genus *Streptomyces* and related actinomycetes using a small number of standardised, reliable tests (Shirling and Gottlieb, 1966). Other investigators made similar attempts at an early form of numerical

taxonomy based on larger numbers of characteristics (Silvestri *et al.*, 1962; Hill *et al.*, 1960) but none achieved a satisfactory classification. A major problem with these early attempts at taxonomic rationalisation was that bacteria labelled as separate strains of the same species based on their original classifications were often grouped into separate clusters.

Chemotaxonomic studies called the previous phylogenies into question but these new data alone could not be used to devise a satisfactory phylogenetically based taxonomy (Embley and Stackebrandt, 1994). The application of molecular techniques, especially 16S rRNA sequencing, to large numbers of actinomycetes, has had a dramatic effect on actinomycete systematics. It was soon discovered that some morphological characteristics given much weight in earlier studies, such as ability to form spores, were not reliable in a phylogenetic system of classification (Stackebrandt *et al.*, 1981). Work is still ongoing in this area but the phylogenetic relationships between most actinomycete genera, and many species, have been established to a degree (See Fig. 1.4), but branching patterns between major clades is still unstable and more sequencing has to be done in order to increase confidence in these relationships (Embley and Stackebrandt, 1994).

1.3.2 Numerical Taxonomy

Numerical Taxonomy is a system of classification which compares characters of each strain with the same characters of every other strain in the study and then places them into related groups on the basis of this comparison. Statistical analysis used for this comparison gives equal weight to a large number of tests (Williams *et al.*, 1983a; Kampfer *et al.*, 1991). Each test should be applicable to all strains to be compared, have a high reproducibility, and the analysis should create groups of low overlap with one another (Sneath 1974, and 1979). Once a "good" classification is achieved it is then possible to weight characteristics as more important depending on their reproducibility and consistency within a cluster (Goodfellow *et al.*, 1992, Williams *et al.*, 1984). This was a radical departure from earlier methods of identification which often placed strains into related groups on the weight of a small number of characteristics the importance of which were subjectively chosen (Pridham and Tresner, 1974). This method is not able to give any reliable phylogenetic information, the close relationships between members of a cluster are phenetically rather than genetically based (Alderson *et al.*, 1984).

The first numerical taxonomic study of actinomycetes was by Silvestri *et al.*, (1962). It applied 100 tests to 200 strains and was the basis for the production of an identification key (Hill and Silvestri, 1962). The results of this study went largely un-used. There followed a number of numerical taxonomic studies on some actinomycete groups, concentrating particularly on the genera *Mycobacterium* and *Nocardia* but with less attention being paid to the problematic sporoactinomycetes, a group in need of taxonomic reform (Alderson *et al.*, 1984).

Williams *et al.*, (1983a) conducted such a study on 475 strains of the genus *Streptomyces*, and related genera which, along with the study by Locci, *et al.*, (1981) became the basis for classification and identification for these groups for the next ten years. The studies were used to define these groups in *Bergey's Manual of Systematic Bacteriology*, vol. 4 (Williams *et al*, 1989). A number of probability matrices were published (Langham, *et al.*, 1989; Williams *et al.*, 1983b) on the basis of the studies by Williams *et al.*, (1983a) and Locci, *et al.*, (1981). These matrices were constructed from those characteristics which showed a high degree of reproducibility and allowed practical application of the information from the studies for the identification and classification of streptomycetes and related genera. A later numerical study published by Williams, *et al.*, (1985), added to the information on genus *Streptoverticillium* .

The study by Williams *et al.*, (1983a) compared morphology; pigmentation; antimicrobial activity of strains against eight test organisms; 11 biochemical tests; 18 degradative tests; antibiotic resistance to 11 compounds; ability to grow at a variety of temperatures, pH levels, salt concentrations and in the presence of a variety of chemical growth inhibitors; growth on 11 sole nitrogen sources and 25 sole carbon sources between each of the strains tested. This was a total of 139 different tests for each strain. Each test was assigned a value of one for a positive result and zero for a negative for the purpose of statistical analysis. On the basis of this analysis the test strains were placed into ten major cluster groups, A to J, at the 77.5 % similarity level. These major clusters were further subdivided into 101 clusters, including 28 single member clusters. A number of species from non-streptomycete genera were included in the test and were distributed within major streptomycete-containing clusters. These included members of the genera *Actinopycnidium, Actinosporangium, Chainia, Elytrosporangium, Microellobosporia, Nocardioides, Saccharopolyspora* and *Nocardiopsis* in cluster-group A, members of the genera *Streptoverticillium,* and *Kitasatoa*

in group F, members of the genera *Actinomadura pelletieri* in group J, other members of the genera *Actinomadura, Microtetraspora* and *Nocardia* in group E. Inclusion of duplicate strains in this test gave a low chance of test error, at 3.36%. The test also gave an indication of the reproducibility of the characters used (Table 1.6).

Table 1.6: Reproducibility of phenotypic characteristics in *Streptomyces*. From Williams, *et al.*, (1983a)

Test	Agreement between duplicates (%)
Presence or absence of spores	100
Spore chain morphology	89.2
Spore chain ornamentation	98.2
Presence or absence of aerial spore mass	95.5
Colour of aerial spore mass	96.4
Presence or absence of diffusible pigments	88.6
pigmentation of diffusible pigments	97.3
pH sensitivity of substrate and diffusible pigment	100
Melanin production on peptone/yeast/iron agar	100
Melanin production on tyrosine agar	95.5
Fragmentation of mycelium	100
Sclerotia formation	100
Sporulation on substrate mycelium	100
Antimicrobial activity mean vs 8 test strains	92.6
Enzymatic activity: mean of eight tests	98.8
ß-Lactamase production on yeast-peptone + glycerol agar	86.4
ß-Lactamase production on Beecham's FS agar	81.8
Production of *Klebsiella* ß-Lactamase inhibitor	97.7
Degradation: mean of 18 substrates	95.3
Antibiotic resistance: mean of 11 antibiotics	95.5
Growth on sole nitrogen source: mean of 11 sources	86.6
Growth on sole carbon source: mean of 25 sources	92.2
Growth at different temperatures and in the presence of chemical inhibitors	93.5

Some characteristics used to group strains in earlier studies were shown to be definitive markers. For example one major cluster was homogeneous for spore colour, but otherwise characters such as spore mass colour, spore

surface ornamentation, and other pigmentation and morphological features were not cluster specific.

Williams *et al.*, (1983a) proposed a rationalisation of actinomycete genera, with *Actinopycnidium, Actinosporangium, Chainia, Elytrosporangium* and *Microellobosporia,* regarded as synonyms of *Streptomyces.* Eventually this proposal was adopted when supported by molecular taxonomic evidence (Goodfellow *et al.*, 1986a to d). Chemotaxonomic data also supported the inclusion of these strains in the genus *Streptomyces* but excluded members of the genera *Nocardioides* and *Saccharopolyspora.*

Some of the clusters defined in the study by Williams *et al.*, (1983a) contained large numbers of strains and it was suggested that further studies were needed to define their relationships and to determine whether the cluster members should be considered as separate species or put together as strains in a large species group.

Similar investigations were conducted for facultatively anaerobic actinomycetes (Holmberg and Nord, 1975; Scholfield and Schaal, 1981), actinomaduras (Goodfellow *et al.*, 1979), rhodococci (Goodfellow and Alderson, 1977), sporoactinomycetes containing *meso-* DAP in the cell wall peptidoglycan (Goodfellow and Pirouz, 1982).

In 1991 a new system of classification based on numerical taxonomy was proposed by Kampfer *et al.*, (1991). The problem with the *Streptomycetaceae* at this time was that there were still a number of species which did not fall into a natural classification (Kampfer *et al.*, 1991) and there was no rapid test for identification of unknown isolates. The matrices constructed based on the results of Williams *et al.*, (1983a) were too large and exacting for ease of use. The use of miniaturized physiological tests may have solved the second of these problems, as it is relatively rapid and easy to use. It was hoped miniaturized tests may help to define the position of strains falling outside the study of Williams *et al.*, (1983a) by surveying a greater number of strains and using a larger number of tests from which further matrices could be made.

The study by Kampfer *et al.*, (1991) compared 821 strains of the genera *Streptomyces* and *Streptoverticillium* using 329 miniaturized tests. Morphological and pigmentation characteristics were not included, as determination of these characters was taken to be too subjective and ill

defined, with difficulties reported in the literature in distinguishing between Retinaculiaperti and Spirales spore chain morphology (Williams and Wellington, 1980; Williams, *et al.*, 1989) and colour of the spore mass (Kutzner, 1981). The tests included carbon source utilization, sugar fermentation, and qualitative enzyme tests using chromogenic substrates. An attempt was made to read the results by photometry but this was not as accurate as visual readings because some colorimetric tests result in a change in substrate colour with little growth of the test organism, giving a false positive. The 821 strains were placed into fifteen major clusters (defined as those with six or more strains), 34 minor clusters (those with less than six strains) and 40 single member clusters. These clusters were defined at the 81.5% similarity level. Two large clusters, 1 and 22, were further divided into morphologically and physiologically distinct cluster-groups on the basis of both numerical data at the 84.0% similarity level and genetic and chemotaxonomic data. Test error was low, 3.11%, comparable to Williams *et al.*, 1983a at 3.36%.

When the clusters obtained by Kampfer *et al.*, (1991) were compared to those of Williams *et al.*, (1983a) there was some agreement, with 76.3% of strains included in both studies grouped into comparable clusters. Many of the minor clusters showed differences but were difficult to compare due to the low numbers within them. There was agreement between the two studies however. Morphological characteristics such as colour of spore mass, as well as being difficult to distinguish, were not homogeneous in any of the clusters and could not be relied upon as phenetic markers. Kampfer *et al.*, (1991) found that many *Streptomyces* deposited in culture collections were assigned to the wrong species and were not accurately described. These findings agreed with those of Williams *et al.*, (1983a), that *Streptomyces* was over-speciated. Both proposed that numerical taxonomy could be used as a method for dealing with this problem. Kampfer *et al.*, (1991) also agreed that the genus *Streptoverticillium* should be regarded as a synonym for *Streptomyces.*

Using the data of Kampfer *et al.*, (1991) as its basis a probability matrix for the identification of Streptomycetes using miniaturized physiological tests has been constructed using 50 of the original 328 tests (Kampfer and Kroppenstedt, 1991). This matrix gives an identification which is 78% likely to be correct.

Numerical taxonomy is a useful tool in differentiating strains and provides valuable phenetic information but it is still necessary to rely on chemotaxonomic and genotypic data to achieve a true phylogenetic classification (Alderson *et al.*, 1984; Kampfer and Kroppenstedt, 1991; Williams *et al.*, 1983a).

1.3.3 Chemotaxonomy

1.3.3.1 Introduction

Chemotaxonomy as defined by Goodfellow and Minniken (1985) is "... the study of chemical variation in living organisms and the use of chemical characters in classification and identification."

Chemotaxonomy was first used in the late 1940's when the development of chromatography and gel electrophoresis made such studies possible (Goodfellow and Minniken, 1985). As analytical techniques became more sensitive and more efficient methods for extraction of cellular components were developed, the importance of chemotaxonomy for bacterial taxonomy was recognised (Lechevalier and Lechevalier 1970). Chemical properties are now a common feature in descriptions of bacterial taxa.

As with numerical taxonomy, chemotaxonomy is not, in its present form, a reliable method of identification to species level (Kampfer *et al.*, 1991; Kroppenstedt, 1985). It is useful as a means of identifying unknown isolates to the generic level (Goodfellow and Cross, 1984; Minnikin and O'Donnell, 1984) (also see Table 1.7) and of placing strains into clusters of related members in order to support and compliment the findings of numerical and molecular taxonomic studies.

Chemotaxonomic markers appear to be more highly conserved than physiological characteristics, and so their contribution to taxonomy may be more phylogenetically based (Sneath, 1989). For example, the clusters based on polar lipid studies for archaea, correspond to those derived from ribosomal RNA-DNA hybridisation studies. These groupings had not been recognised using morphological and physiological characters (Goodfellow and Minnikin, 1985). Chemotaxonomic methods have contributed to the redefinition and reorganisation of taxa and have been useful in the indication of anomalies within existing taxa (Collins *et al.*, 1982; Collins and Jones, 1980).

Chemotaxonomy can be a relatively quick method for differentiation between strains. It has been suggested that the limits on how quickly strain may be analysed by chemotaxonomy are set by the time required to obtain sufficient biomass (O'Donnell *et al.*, 1985). This is not strictly true in all cases. The proportions of isoprenoid quinones, for instance, have been shown to vary with the growth cycle, and for these compounds it is necessary to take samples of the microorganism at defined stages of growth (Saddler *et al.*, 1986). For fatty acids, the variation in profiles is negligible through logarithmic and stationary growth phases (Verma and Khuller, 1981; Saddler *et al.*, 1986) but has been shown to vary with temperature of incubation (Finotti *et al.*, 1993). Other factors which have been shown to affect a range of chemotaxonomic markers include: pH, nutrient levels, accumulating metabolic product, growth rate of microorganism, nature of carbon and nitrogen sources, oxygen levels, trace metals, and light levels for phototrophs (Ratledge and Wilkinson, 1988a). It is therefore important to standardize growth conditions and where possible to avoid the use of complex growth media (Ratledge and Wilkinson, 1988a).

Screening a large number of strains using chemotaxonomy for comparison amongst the strains or with a database is quick and easy when compared to numerical taxonomy. Chemotaxonomic methods are and usually cheaper to perform than molecular taxonomic methods. For large numbers of environmental isolates chemotaxonomy can be the best option for quick determination of genera or as a means of grouping closely related isolates.

Chemical markers commonly used in bacterial chemotaxonomy include isoprenoid quinones, lipid soluble pigments, lipoteichoic acids, polar lipids (including fatty acids, Isoprenoid ethers and other long chain components), proteins, peptidoglycans, polysaccharides, teichoic acids, lipopolysaccharides, bound lipids and free lipids (including glycolipids, sulphoglycolipids and waxes) (Goodfellow and Minniken, 1985; Ratledge and Wilkinson, 1988a).

The value of different chemical characters varies between taxa. For example, isoprenoid quinone distribution in endospore forming aerobic bacteria and polar lipid patterns in staphylococci are fairly uniform, but these same compounds can be used in members of *Bacillus* and *Staphylococcus* for sub-generic classification (Goodfellow and Minnikin, 1985). The chemical characteristics which have been most widely studied in the actinomycetes and proved most useful in classification are shown in Table 1.7.

Table 1.7: Suprageneric groups of actinomycetes and some of their chemical properties. (From Goodfellow, 1989)

Group/genus	Wall chemo-type[a]	Whole cell sugar pattern[b]	Peptido-glycan type[c]	Fatty acid pattern[f]	Major menaquinone[d]	Phospho -lipid type[e]	Mol% G+C of DNA
ACTINOBACTERIA							
Agromyces	VII	-	B2γ	2c	-11, -12, -13	PI	71-77
Aureobacterium	VIII	-	B2β	2c	-11, -12	PI	65-76
Clavibacter	VII	-	B2γ	2c	-9, -10	PI	68-75
Curtobacterium	VIII	-	B2β	2c	-9	PI	68-75
Microbacterium	VI	-	B1', B2β	2c	-11, -12	PI	60-75
Arthrobacter	VI	-	A3α	2c	-9(H_2)	PI	59-70
Micrococcus	VI	-	A3α	2c	-7(H_2), -8(H_2), -9(H_2)	PI	64-75
Renibacterium	VI	-	A3α	2c	-9	PI	53-54
Rothia	VI	-	A3α	2c	-7	PI	54-57
Stomatococcus	VI	-	A3α	ND	ND	PI	56-60
Cellulomonas	VIII	-	A4β	2c	-9(H_4)	PV	71-76
Oerskovia	VI	-	A4α	2c	-9(H_4)	PV	70-75
Promicromonospora	VI	-	A4α	ND	-9(H_4)	PV	70-75
Actinomyces	V, VI	-	A4α, A4β	1a, 1c	-10(H_2, H_4)	PII	57-69
Arcanobacterium	VI	-	A5α	1a	-9(H_4)	ND	48-52
Arachnia	I	-	A3γ'	2c	-9(H_4)	PI	63-65
Pimelobacter	I	-	A3γ	2a	-8(H_4)	PI	69-74
Brevibacterium	III	C	A1γ	2c	-8(H_2)	PI	60-67
Dermatophilus	III	B	A1γ	1a	-8(H_4)	PI	57-59
ACTINOPLANETES							
Actinoplanes	II	D	A1γ'	2d	-9(H_4), -10(H_4)	PII	72-73
Ampullariella	II	D	A1γ	2d	-9(H_4), -10(H_4)	PII	72-73
Catellatospora	II	D	ND	ND	-9(H_4), -10(H_8)	PII	71-72
Dactylosporangium	II	D	A1γ	3b	-9(H_4, H_6, H_8)	PII	71-73
Micromonospora	II	D	A1γ	3b	-9(H_4), -10(H_4)	PII	71-73
Pilimelia	II	D	A1γ	2d	-9(H_2, H_4)	PII	ND
MADUROMYCETES							
Actinomadura pusilla group	III	B, C	A1γ	3c	-9(H_0, H_2, H_4)	PIV	64-69
Microbispora	III	B, C	A1γ	3c	-9(H_2, H_4, H_6)	PIV	67-74
Microtetraspora glauca group	III	B, C	A1γ	3c	-9(H_0, H_2, H_4)	PIV	66
Planobispora	III	B	A1γ	3c	-9(H_2, H_4)	PIV	70-71
Panomonospora	III	B	A1γ	3c	-9(H_2)	PIV	72
Streptosporangium	III	B	A1γ	3c	-9(H_2, H_4)	PIV	69-71
MICROPOLYSPORAS							
Actinopolyspora	IV	A	A1γ	2c	-9(H_4, H_6)	PIII	64
Amycolata	IV	A	A1γ	3e	-8(H_4)	PIII	66-72
Amycolatopsis	IV	A	A1γ	3f	-9(H_2, H_4)	PII	66-69
Faenia (Micropolyspora)	IV	A	A1γ	2c	-9(H_4)	PIII	66-71
Kibdelosporangium	IV	A	A1γ	3c	ND	PII	66
Pseudonocardia	IV	A	A1γ	2b	-8(H_4)	PIII	79
Saccharomonospora	IV	A	A1γ	2a	-9(H_4)	PII	69-74
Saccharopolyspora	IV	A	A1γ	2c	-9(H_4)	PIII	77
MULTILOCULAR SPORANGIA							
Frankia	III	B, C, E	ND	1	ND	PI	66-71
Geodermatophilus	III	C	A1γ	2b	9(H_2)	PII	73-76

Group/genus	Wall chemotype[a]	Whole cell sugar pattern[b]	Peptido-glycan type[c]	Fatty acid pattern	Major menaquinone[d] /MK	Phospho -lipid type[e]	Mol%G +C of DNA
NOCARDIOFORMS							
Caseobacter	IV	A	A1γ	1b	-8(H_2), 9(H_2)	ND	65-67
Corynebacterium	IV	A	A1γ	1a	-8(H_2), 9(H_2)	PI	51-63
Mycobacterium	IV	A	A1γ	1b	-9(H_2)	PII	62-69
Nocardia	IV	A	A1γ	1b	-8(H_4), 9(H_2)	PII	64-72
Rhodococcus	IV	A	A1γ	1b	-8(H_2), 9(H_2)	PII	63-72
NOCARDIOIDES							
Nocardioides	I	-	A3γ	3a	-8(H_4)	PI	ND
STREPTOMYCETES							
Intrasporangium	I	-	A3γ	1aND	-8	PI	ND
Kineosporia	I	-	A3γ	ND	-9(H_4)	PIII	ND
Sporichthya	I	-	A3γ	3a	-9(H_6, H_8)	ND	ND
Streptomyces	I	-	A3γ	2c	-9(H_6, H_8)	PII	69-78
Streptoverticillium	I	-	A3γ	2c	-9(H_6, H_8)	PII	69-73
THERMOMONOSPORAS							
Actinomadura madurae group	III	B	A1γ	3a	-9(H_6)	PI	66-69
Actinosynnema	III	C	ND	3f	-9(H_4), 10(H_4)	PII	71-73
Microtetrospora viridis	III	C	ND	3a	-9(H_4)	PI	67
Nocardiopsis	III	C	ND	3d	-10(H_2,H_4, H_6)	PIII	64-69
Saccharothrix	III	C	ND	3f	-9(H_4), -10(H_4)	PII	70-76
Streptoalloteichus	III	C	ND	ND	-9(H_6), -10(H_6)	ND	ND
Thermomonospora	III	C/C	ND	3e/3c	-9(H_2,H_4)/-10(H_4, H_6)	PII/PIV	ND/ND
OTHER GENERA							
Glycomyces	II	D	ND	2c	-9(H_4), -10(H_4)	PI	71=73
Kitasatosporia	I, III	C	ND	ND	ND	ND	66-73
Spirillosporia	III	B	A1γ	3a	-9(H_4, H_6)	PI, PII	69-73
Thermoactinoyces	III	C	A1γ	2b	-7, -9	ND	53-55

[a] Major constituents in wall chemotypes are: I , L-DAP and glycine; II, *meso* -DAP and glycine; III, *meso* -DAP; IV, *meso* -DAP, arabinose and galactose; V, lysine and ornithine; VI, variable presence of aspartic acid and galactose; VII, diaminobutyric acid and glycine, with variable lysine; and VIII, ornithine.

[b] Whole cell sugar patterns are: A, arabinose and galactose; B, madurose; C, no diagnostic sugars; D, arabinose and xylose; and -, not applicable.

[c] Numbers refer to variations of cross linkage. Greek letters mark diversity of amino acids in position three of the peptide side chain and prime (') indicates the replacement of alanine in position 1 in group A peptidoglycans by glycine.

[d] The large numeral refers to number of isoprene units and in (H_x), x = the number of units hydrogenated.

[e] Characteristic Phospholipids are: PI, phosphatidylglycerol (variable); PII, phosphatidylethanolamine only; PIII, phosphatidylcholine (with phosphatidylethanolamine, phosphatidylmethylethanolamine and phosphatidylglycerol variable, no phospholipids containing glucosamine); PIV, phospholipids containing glucosamine (with phosphatidylethanolamine and phosphatidylmethylethanolamine variable); and PV, phospholipids containing glucosamine and phosphatidylglycerol.

[f] From Kroppenstedt, 1985.

As can be seen in Table 1.7, no one chemotaxonomic marker is sufficient for identification of all strains of actinomycetes to genus level. Major menaquinones are the best distinguishing characteristic, and 15 out of the 63 genera may be delineated with this one characteristic. Most markers are evenly spread through a number of suprageneric groups however, and a combination of two or more chemotaxonomic methods are usually necessary for identification of genera.

1.3.3.2 Wall Chemotype

Determination of the cell wall chemotype of a strain has been developed as a rapid method in chemotaxonomy. It combines the presence or absence of two classes of compound in the cell wall to give a wall chemotype. These compounds are cell wall sugars and diamino acids. Purified cell wall material can be prepared by an alkali treatment of whole cells and identified by thin layer chromatography with appropriate standards (Bousefield *et al.*, 1985).

Diamino-acids in the cell walls of actinomycetes as major constituents include L-Diaminopimelic acid (L-DAP), *meso*-DAP, ornithine, lysine, diaminobutyric acid (DAB), glycine and aspartic acid. Cell wall sugars, in actinomycetes include galactose and arabinose. Different combinations of these are characteristic of actinomycete genera (see Table 1.7, note a).

Cell wall chemotypes are useful for the identification of the "coryneform" bacteria (or actinobacteria) as there is some variation in chemotype between genera. Cell wall chemotypes conform exactly to supra-generic groupings for all other actinomycetes (Goodfellow, 1989). They confirm the groups based on other taxonomic criteria, but are of little use as a method of identification to genus level. Cell wall chemotypes have also proved useful in indicating possible errors in classification where members of the same genus have different peptidoglycan types (Collins and Jones, 1980).

1.3.3.3 Whole Cell Sugar Pattern

Sugars occur in bacterial cells as components of nucleic acids, capsular polysaccharides and lipopolysaccharides (Jantzen and Bryn, 1985). Extraction is by a relatively simple process involving cell disruption, methanolysis,

hexane extraction of fatty acids and analysis of the remaining sugars by gas chromatography (Jantzen and Bryn, 1985).

Within the Actinomycetes, diagnostic sugars are usually simple mono- or di- saccharides. They are specific for some groups, for example the combination of arabinose and galactose occurs only in micropolysporas and nocardiaforms, and arabinose and xylose in actinoplanetes (Goodfellow, 1989) but other sugar patterns are spread through a number of groups and are useful for classification to the generic level only in combination with other taxonomic data.

1.3.3.4 Peptidoglycan Type

Most bacteria are enveloped by a protective cell wall, the main component of which in both Gram positive and Gram negative bacteria is peptidoglycan (Schleifer and Seidl, 1985). Peptidoglycan is a polymer which is made from strands of polysaccharide cross-linked through short peptide chains (Schleifer and Seidl, 1985).

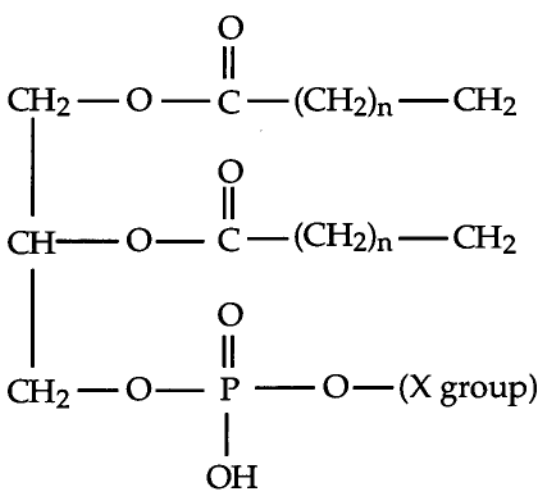
There are a number of variations in structure of peptidoglycans between different taxa which consists of differences in amino acid sequences of the linking peptide chains and differences in the mode of cross linking between these chains (Schleifer and Seidl, 1985). Cross linkage can be either via the amino acid in position three of one chain and position four of the other, designated "group A" peptidoglycan, or between the amino acid in position two of one chain and position four of the other, designated "group B" peptidoglycan (Schleifer and Kandler, 1972). The peptide chain is four or five amino acids in length and varies between three different amino acids at position one, five amino acids at position two and 11 amino acids at position three (Schleifer and Seidl, 1985). These structural variations have been codified (Schleifer and Kandler, 1972) as shown in Table 1.7 (note [c]).

Within the Actinomycetes, peptidoglycan type is rarely a definitive characteristic of a genus, with some exceptions such as members of genera *Actinoplanes* (A1γ), *Arachnia* (A3γ) and *Arcanobacterium* (A5α). It can be useful for identification to generic level in combination with other taxonomic data. As determination of peptidoglycan type can be an involved process however (Schleifer and Seidl, 1985) it may not be as useful as some other chemotaxonomic techniques for rapid screening of large numbers of isolates.

1.3.3.5 Phospholipid Type

Phospholipids are present in the bacterial cell where they are the basic unit of bilayer membranes. Phospholipids consist of a glycerol primary unit, two hydrophobic fatty acids and a hydrophilic phosphate ester sometimes referred to as the "X group" (Brock*et al.*, 1984). It is this X group which defines the phospholipid type and those which are used for characterisation in actinomycetes include glycerol, ethanolamine, choline, methylethanolamine and glucosamine (Goodfellow, the prokaryotes).

Figure 1.6: Primary structure of phospholipid molecule. X group denotes position filled by a phosphate ester of a non-fatty acid molecule.



Phospholipid type correlates to a limited extent with supra-generic groups, for instance the Maduromycetes are all type IV, the only actinomycete group with this pattern, and the Actinoplanetes type II, though a number of non-Actinoplanetes share this pattern. Phospholipid types are not genus specific but are useful for classification in combination with other taxonomic data.

Early reports of phospholipid patterns in a number of actinomycete genera (Lechevalier *et al.*, 1977) indicated discrepancies in classification and helped to redefine various taxa.

1.3.3.6 Menaquinones

Isoprenoid quinones are a class of terpenoid lipids that occur in the plasma membrane of bacteria where they have a role in electron transport, oxidative phosphorylation and possibly active transport (Collins and Jones, 1981). They will degrade rapidly in the presence of oxygen and strong light and also in alkaline conditions and are usually extracted in an atmosphere of nitrogen in a container shielded from strong light (Collins, 1985a).

Studies by Bishop *et al.*, (1962), Lester and Crane (1959), and Page *et al.*, (1960) indicated the potential of isoprenoid quinones for chemotaxonomy. This potential was not exploited for over a decade, when bacterial chemotaxonomists began to compile information on the variations in structure of isoprenoid quinones between different microorganisms (Collins and Goodfellow, 1979; Collins *et al.*, 1980; Collins and Jones, 1979; Collins *et al.*, 1977; Collins *et al.*, 1981; Collins *et al.*, 1980; Watanuki and Aida, 1972; Yamada *et al.*, 1968; Yamada *et al.*, 1976; Yamada *et al.*, 1977a; Yamada *et al.*, 1977b) and this data continues to be collected (Alderson *et al.*, 1985; Amandi and Alderson, 1991; Athalye *et al.*, 1984; Collins *et al.*, 1985; Kroppenstedt, 1985).

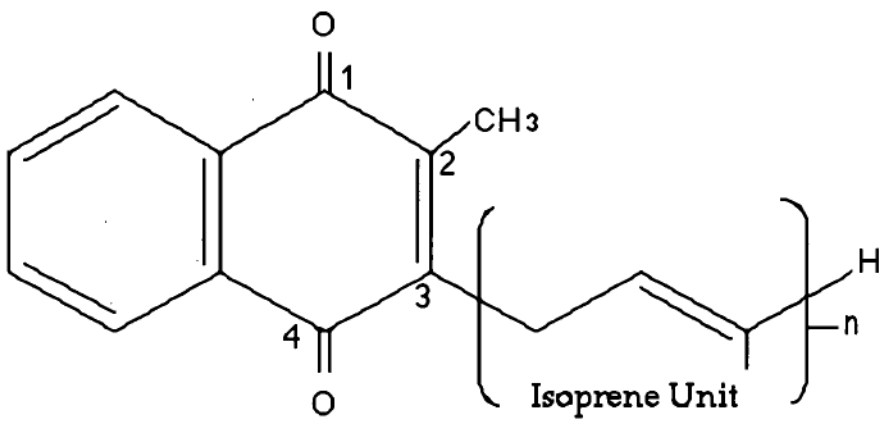
In 1981 a review by Collins and Jones assembled the available information on the distribution of isoprenoid quinones in bacteria. Five different basic structures had been identified, phylloquinones, menaquinones, chlorobiumquinones, demethylmena-quinones, plastiquinones, and ubiquinones. There was a correlation between the type of isoprenoid quinone a species produced and the taxa into which it had been placed based on other taxonomic data.

Yokata *et al.*, in 1992 incorporated all available data on quinone patterns of the Gram negative eubacteria into a single table, a total of 174 publications. The data contained in this table supported the conclusions of Collins and Jones (1981), that closely related species had the same quinone pattern.

The structure of the basic quinones can vary widely between strains, especially in the menaquinones. The C-3 multiprenyl side chain of menaquinones (see Fig. 1.7) can vary in length from one to 15 isoprene units (Collins and Jones 1981), and this chain can also be saturated to varying degrees. This variation is usually represented in abbreviated form,

for example a menaquinone with nine isoprene side units, one of which was saturated would be represented: MK-9(H_2). Other isoprenoid quinones also vary in the length of the side chain but no variation in saturation of the isoprene units has been reported.

Fig. 1.7 Structure of Menaquinone, from Collins and Jones (1981)



Actinomycetes, except for a few taxa of uncertain classification (Collins and Jones, 1981), contain menaquinones and it is possible to distinguish between many actinomycete genera on the basis of menaquinone profile alone (see Table 3.3.6). Actinomycete menaquinones are easily extracted and analysed by a variety of methods (Collins, 1985a; Collins, 1985b; Collins *et al.* , 1977; Collins, 1982; Tamoaka *et al.*, 1983, Minnikin, *et al.*, 1984; Tamaoka, 1986; Dunphy, *et al.* , 1971), and the menaquinones of many genera have been studied in detail (Yamada *et al.* , 1968; Yamada, *et al.* , 1976; Yamada, *et al.* , 1977a; Yamada, *et al.* , 1977b; Collins *et al.* , 1982; Collins *et al.* ,1977; Collins, 1986; Collins, and Goodfellow, 1979; Collins, *et al.* , 1980; Collins *et al.* , 1985; Amandi and Alderson 1991; Athalye *et al.* , 1984; Alderson *et al.* , 1985) so menaquinones are useful markers for identifying members of this taxa to genus level.

1.3.3.7 Fatty Acids

Fatty acids are precursors for the synthesis of most lipids in bacteria (Verma and Khuller, 1983) with the general formula $CH_3(CH_2)nCO_2H$ (Ratledge and Wilkinson, 1988). They are not usually present as free fatty acids, but rather as components of triglycerides, phospholipids, and other lipids in bacterial plasma and cellular membranes (Ratledge and Wilkinson, 1988b; Goodfellow and Minnikin, 1985). They consist of a backbone of carbon atoms which vary in length from C_8 to C_{26} (though more usually in the range C_{14} to C_{20}, Ratledge and Wilkinson, 1988), they may be hydroxylated or not, fully or partially saturated, may include a cyclopropane ring or more rarely a cyclopropene ring, may have methyl branches, usually one or two, in either the iso- or anteiso- position, and may include combinations of a number of these features (Jantzen and Bryn, 1985; Kroppenstedt, 1985; Ratledge and Wilkinson, 1988). In addition, some actinomycetes have a high molecular weight fatty acid component, the mycolic acids (C_{14} to C_{20}, Ioneda, 1984) which are found in members of the genera *Mycobacterium*, *Corynebacterium*, *Rhodococcus* and *Nocardia* (Jantzen and Bryn, 1985; Brennan, 1988).

Fatty acids are released by saponification and the fatty acids are then methylated to form fatty acid methyl esters (Kroppenstedt, 1985). These can be extracted with organic solvents and a profile obtained by gas chromatography. The identity of the peaks in the profile is determined by mass spectrometry (Kroppenstedt, 1985).

The variations between different taxa in fatty acid profiles can be a diagnostic feature. Once an actinomycete strain has been identified to the supra-generic level, a fatty acid profile is useful for further identification in all supra-generic groups except the maduromycetes. This is the only supra-generic group to show homogeneity in fatty acid patterns between genera (Table 1.7).

Table 1.8: Fatty acid patterns among members of the order *Actinomycetales.* (Kroppenstedt, 1985).

	Saturated	Un-saturated	Iso 14/16 and 18	Iso-15/17	Anteiso-15/17	10-methyl-17	10-methyl-18	Cyclo-propane
Corynebacterium	+++	+++	--	--	--	--	--	--
Mycobacterium, Nocardia	+++	+++	--	--	--	--	++	--
Actinomyces israelii	+++	+++	--	--	--	--	--	++
Saccharomonospora	++	+	+++	+	(+)	--	--	--
Thermoactinomyces	(+)	+	++	+++	+	--	--	--
Streptomyces	+	var.	+++	+	+++	--	--	--
Actinoplanes	+	+	+++	+++	+++	--	--	--
Actinomadura madurae	+++	++	+++	(+)	(+)	(+)	+++	--
Micromonospora	+	+	+++	+++	++	++	(+)	--
Actinomadura pusilla	+	+	++	+	+	+++	(+)	--
Nocardiopsis	+	+	+++	++	+++	(+)	+++	--

Fatty acid patterns can be diagnostic for a particular actinomycete genus (table 1.7 above) in some cases or when used in combination with other morphological, physiological or chemotaxonomic data. Numerical comparison of fatty acid profiles is being increasingly used to identify bacteria to genus level in combination with other techniques (Bousefield *et al.,* 1983; Kroppenstedt, 1985) and has already proved useful in this role in the study of Antarctic bacteria (Tearle and Richard, 1987).

Fatty acid profiles can also vary within a genus. The major components as shown in Tables 1.7 and 1.8 are always present, but presence or absence of minor components and the variation in proportions of all fatty acids between strains is constant under standard growth conditions (Bowers *et al.,* 1995). It is possible to establish a library of fatty acid profiles which can be used for identification. One problem with this technique is that methods of fatty acid extraction and analysis are rarely consistent enough between laboratories to allow comparisons of profiles between them so a centralised

library of strain profiles is not practical (Kroppenstedt, personal communication, 1996). Such a library of profiles can however be a useful tool for rapid identification of strains in ecological studies (Ratledge and Wilkinson, 1988b; Bowers *et al.*, 1995).

Strain specific differences in fatty acid profiles can also place large numbers environmental isolates into clusters of similar or identical strains. This saves on further attribute testing especially when a narrow range of microorganisms are being isolated, for which morphological and physiological characters are of little differential value.

A further use for fatty acid profiles is in monitoring changes in microbial communities over time, by extraction and comparison of fatty acids of whole populations (Ratledge and Wilkinson, 1988b; Presting *et al.*, 1993).

Fatty acid profiles are often used in polyphasic taxonomic studies of actinomycetes and tend to support molecular based phylogenies. For example members of the genera *Dermatophilus* and *Geodermatophilus* are morphologically very similar but can be separated on the basis of fatty acid profiles, which agrees with findings based on molecular techniques (Stackebrandt *et al.*, 1983).

1.3.4 Molecular Taxonomy: Nucleic Acids

1.3.4.1 Introduction

Molecular taxonomy is based on variations in nucleic acid sequences between different bacteria. The methods used could properly fall under the heading of chemotaxonomy but because of the impact molecular techniques have had on taxonomy they are reviewed here separately.

Before the advent of molecular taxonomy, a true phylogenetic classification of bacteria was a difficult proposition, and considered by many to be impossible (Stainer and van Niel in Olsen *et al.*, 1986). In 1965 Zuckerland and Pauling published an article arguing that variations in molecular structure are a record of evolutionary history. In the same year Woese published an article on the evolution of the genetic code. It was not until the end of the 1970's however that molecular techniques were recognised as useful tools for studying the phylogenetic relationships of bacteria and were applied in the classification of prokaryotes (Olsen *et al.*, 1986).

In order to use molecular techniques for phylogenetic studies it was necessary to use a molecule or series of molecules which are unaffected by evolutionary pressures and which change at a relatively constant rate, so that degree of difference between molecules can be used as an accurate measure of evolutionary distance. A protein or nucleic acid whose sequence changes randomly over time can be considered a molecular clock (Woese, 1987), and if its rate of change can be determined, it is possible to use it to determine when divergence occurred between two organisms. It is also necessary that the rate of change of the molecule be applicable to the time of divergence between the organisms being compared.

At the end of the 1970's, studies on the structure and conserved nature of the 16S ribosomal RNA molecules indicated that they might be an ideal for this role (Fox and Woese, 1975) and it was later suggested that the 16S rRNA molecule was the most practical of the fragments for broad based phylogenetic studies (Fox *et al.*, 1977).

Ribosomes are essential to protein formation and fulfil this role in all cellular organisms (Fox *et al.*, 1980). They are assumed to have developed early in evolutionary history and because of their vital role they are functionally and structurally constrained. Much of the sequence of rRNA molecules is more highly conserved than those sections of the bacterial genome that code for proteins (Fox, *et al.*, 1980). Some changes do occur however. Change is slower in the areas under functional constraint and faster in those areas in which change does not appear to affect structure and function of the ribosome. These patterns of differing rates of variation appear to be consistent throughout the eubacteria (Woese, 1987). These patterns of variation means rRNA comparisons can reveal phylogenetic relationships over long evolutionary periods using conserved regions, and over shorter times using comparisons of the highly variable regions.

rRNA sequencing can also provide a means of measuring the distance between organisms in real time based on the rate of change in these sequences. The rate of base substitution in rRNA sequences has been calculated by comparison of the fossil record with sequence divergence in closely related obligate endosymbiotic bacteria. Two studies gave a roughly equivalent result, suggesting that this rate of change was around 1-2% per 50 million years (Ochman and Wilson, 1987; Moran *et al.*, 1993).

rRNA has a number of other inherent benefits for taxonomic studies: The size of rRNA fragments (especially the 16S and 23S) contains sufficient information to allow statistically significant comparisons (Fox *et al.*, 1980); there seems to be no exchange of genetic code between different organisms in this area of the genome so a true evolutionary comparison can be made (Fox *et al.*, 1980); ribosomes represent a large proportion of bacterial cell mass and so techniques of identification based on sequence differences are made easier (Fox *et al.*, 1980).

By the early eighties, rRNA-based phylogenetic studies began to have an impact on bacterial classification. The major lines of prokaryotic descent as presented by Fox *et al.*, (1980) showed that many physiological features and some characters such as morphology had no phylogenetic basis while other characters were reported to be more or less reliable in this respect.

Although in many cases taxa defined by molecular techniques matched those produced by chemotaxonomic and numerical taxonomic comparisons (Woese, 1987), there were numerous exceptions, and the advantages of a phylogenetic approach to taxonomy have been questioned. Sneath (1989) argued that it is the place of taxonomy to give a system of classification which is "useful to a range of scientific purposes, including identification, and to produce data bases which summarize relevant information about organisms", and also that the taxonomic arrangement should produce groups whose members share many common properties and about which many generalisations can be made. Sneath said that there is "not necessarily a connection between phenetic content and historical origin", and if a the groups defined by a phylogenetically based taxonomy cannot be shown to share common properties then this system will have little use outside of academic interest.

It is true that initially some groups formed by rRNA sequence comparisons defied phenotypic understanding (Woese, 1987). However this concern over a lack of consistency in fundamental properties may betray a blinkered attitude which does not ask the question: what are fundamental properties? Decades of use appear to have given some characteristics such as Gram stain, autotrophy/heterotrophy divisions, and more obvious morphological characteristics a legitimacy they did not necessarily rate. For example, only after the archaebacteria had been defined by rRNA studies was it discovered that their membrane lipids were distinctive (Krieg, 1988). The same is true of the actinomycetes where parallel developments in chemotaxonomy and

numerical taxonomy have tended to support and define phenotypically those taxa which had been identified genotypically.

There are a number of advantages to a phylogenetically based system of classification for eubacteria. Before molecular techniques were employed many traditional genera described by a small set of phenotypic characters were poorly defined (Stackebrandt, 1988). Numerical taxonomy went some way towards solving this problem, but although this system tried to determine the correct weight of the defining characteristics used there were still major differences in the clusters determined between different studies (compare Williams *et al.*, 1983a and Kampfer *et al.*, 1991 in Section 1.3.2). As a result there was frequent shifting and re-classification after each investigation of a new phenotypic characteristic and a difficulty in assigning new species to their correct taxa. Some genera such as the genus *Streptomyces* became cluttered with poorly defined new species. Other genera consisted of strains lumped together with no acknowledged fundamental properties, save their shared lack of any, such as occurred with many species of the genus *Pseudomonas*. A phylogenetically based system is not subject to constant re-arrangement or accidental assignment or wrongful identification of strains because it is based upon evolutionary relationships (Krieg, 1988).

Methods based on molecular techniques have already demonstrated their use in microbial ecology. Regions of rRNA molecules which contain sites unique to genera or species have provided good target sites for oligonucleotide probes and site specific enzymes. These methods are rapid and reliable and have allowed advances in understanding of microbial ecology that was previously not possible using traditional techniques.

A nucleic acid based system is also useful for defining taxonomic ranks (Stackebrandt, 1988). Decisions on the boundaries for designation of taxa as a species, genera, etc. cannot be made on one character alone but a combination of 16S rRNA sequencing, DNA:DNA homology, protein sequences and other molecular techniques can help define them more clearly.

It has been pointed out that all statistical methods of nucleic acid sequence comparisons are based on certain assumptions about evolutionary processes. Phylogenies are often based on sequence variations in a limited number of positions whose rate of change may not be consistent and

therefore should be weighted differently. It is also impossible to tell whether or not a single site may have changed a number of times since two strains have diverged (Felsenstein, 1988). Therefore, despite the value of 16S rRNA sequences, a combination of techniques is always necessary when defining taxa.

The definition of the Actinomycetes has undergone considerable revision under the impact of molecular techniques. It now includes many groups which were not formerly considered to be closely related such as *Micrococcus, Renibacterium, Stomatococcus* and *Acidothermus* and others which were only thought to be loosely related such as the coryneforms, *Arthrobacter* and their relatives, and the propionobacteria (Embley and Stackebrandt, 1994).

Change has taken place at the generic and specific level as genotypic data has confirmed relationships suggested by numerical taxonomy and chemotaxonomy or has revealed new relationships. For example, the genera *Streptoverticillium* and *Streptomyces* , thought for a number of years to be members of the same genus on the basis of numerical studies (Williams *et al.,* 1983a) were confirmed in this relationship on the basis of DNA:DNA hybridization and 16S rRNA sequence data (Witt and Stackebrandt, 1990). Similarly for the genera *Kitasatosporia,* and *Streptomyces* (Wellington *et al.,* 1992b). Molecular techniques have had large impact on bacterial taxonomy but taxa are still usually described and defined on the basis of a number of different taxonomic characteristics (see section 1.3.6).

1.3.4.2 Guanine and Cytosine mol%

The percentage of guanine and cytosine in the whole genome (G+C mol%) can be used in combination with other techniques to identify bacteria to genus level. G+C mol% has been used as a taxonomic marker since the late 1960's (Krieg, 1988) and was included in the 1974 edition of *Bergey's Manual of Determinative Bacteriology.* The ratio of A+T to G+C varies from 23% to 78% in different taxa (Goodfellow and Minnikin 1985). G+C mol% also has application in developing a phylogenetic taxonomy, for instance, amongst the Gram positive bacteria, all species are separated into two major taxa on the basis of whether they have high or low G+C mol% (Woese, 1987).

Generally, if two strains differ by greater than 5% in G+C they are not in the same species and if they vary by greater than 10% they are not in the same genus. However, if two bacteria have the same G+Cmol% it does not necessarily mean that they are closely related (Krieg, 1988). This ratio is also important in DNA:DNA homology studies as DNA:DNA hybridization will not occur if the G+C mol% of the strains to be compared differs by greater than 10 to 20% (Krieg, 1988).

1.3.4.3 DNA:DNA Similarity

If double stranded DNA in solution reaches a certain temperature it denatures or melts. If the solution is cooled the DNA will then re-associate and become helical again at a specific temperature. These changes in state can be detected by a number of methods. If the DNA of two strains are combined in equal proportion, denatured, and allowed to re-associate at a specific temperature variations in sequence between the two will cause the time taken for the DNA to re-associate to be greater than that for re-association of double stranded DNA of a single species (Bradley, 1980). This difference in re-association time can be used to determine degree of similarity of genomic DNA between two strains.

In an attempt to define the concept of the term "species" for bacteria, it was proposed by Wayne *et al.*, (1987) that any two strains of bacteria with greater than 70% similarity in DNA:DNA hybridization studies would be considered the same species. At levels below this, the assigning of exact phylogenetic relationships is probably not possible. Similarities of between 30 and 65% may indicate close relatives but this must be correlated with other taxonomic data (Owen and Pritcher, 1985). The term similarity does not refer to degree of sequence homology. A similarity of 50% in DNA:DNA hybridization between two strains indicates a sequence homology of approximately 90 to 95%, and those strands which are capable of hybridization at all are unlikely to have sequence differences of greater then 10 to 20% (Stackebrandt and Liesack, 1993). Relative DNA similarity values are only comparable between very closely related organisms, as it is not possible to find a linear correlation between sequence homologies and hybridization values (Stackebrandt and Liesack, 1993).

This method is therefore best suited for determining phylogenetic relationships between closely related bacteria, at the species or generic level

(Bradley, 1980; Stackebrandt and Schleifer, 1984) where other taxonomic methods used to determine phylogeny are often uncertain (Fox *et al.*, 1992).

1.3.4.4 Ribosomal rRNA Sequencing

As noted in section 3.4.1, the variation in ribosomal RNA has been of central importance for our new understanding of bacterial phylogenetics. Ribosomal fragments occur in three sizes, 5S, 16S and 23S in prokaryotes. Sequence variation in rRNA used to be determined by a procedure known as oligonucleotide cataloguing. rRNA was digested into short fragments and those greater than 5 bases long were fully sequenced. This method would cover about 30-40% of the bases in the 16S rRNA molecule and was good for defining major phyla but was not so useful for subdivisions. It was also costly and technically difficult (Embley and Stackebrandt, 1994). In 1985 a much faster and simpler method 16S rRNA sequencing was published (Lane *et al.*, 1985) and now it is possible to obtain whole sequences with relative ease through PCR of the ribosomal genome (rDNA) and direct the sequencing of product with DNA polymerase.

5S rRNA is easily obtained and sequenced (Stackebrandt and Leisack, 1993) and can be used to determine some phylogenetic relationships, but because of its small size (about 120bp) any errors can effect results markedly and variations in sequence often seem to give a distorted view of phylogenies (Stackebrandt and Leisack, 1993) because of their larger proportion of the total code as compared with variations in 16S and 23S rRNA sequences.

23S rRNA sequence comparisons result in phylogenetic trees which are in good agreement with those resulting from 16S rRNA comparisons. 23S rRNA sequences are longer than 16S rRNA (~1600bp :~3000bp, Olsen *et al.*, 1986) and therefore take longer to analyse and are more expensive to carry out. They are useful as a means of confirming and clarifying phylogenies based on 16S sequences but extensive 23S rRNA sequencing does not appear to be worthwhile for the information it yields.

16S rRNA is now a standard technique used in the description of many new species of bacteria both as an aid to identification and as a partial justification for the naming of a particular strain as a new species. Comparison of 16S rRNA sequences has had enormous impact on our understanding or bacterial phylogenetic relationships. It has also allowed the development of a number of techniques for rapid identification based

on the specificity of particular sequences for particular genera or species (see 1.3.4.5, 1.3.4.6, 1.3.4.7 and 1.3.4.8 below).

There have been a number of good reviews on the impact and use of 16S rRNA on bacterial taxonomy in general (Woese, 1987; Gutell *et al.*, 1994; Ludwig and Schleifer, 1994; Felsenstein, 1988) and actinomycetes specifically (Embley and Stackebrandt, 1994; Stackebrandt and Schliefer, 1984; Stackebrandt *et al.*, 1985).

16S rRNA sequences are not always as useful as they might appear for identification of species. As pointed out by Fox *et al.*, (1992) some errors are inherent in the system of sequence analysis and a sequence homology of greater than 99% may or may not indicate that two organisms are members of the same species. DNA:DNA hybridization studies are usually required when sequences are so similar. 16S rRNA sequence comparisons are most applicable in determining inter and intra-generic phylogenetic relationships but not usually lower taxa.

16S rRNA sequencing has not only changed our view of taxonomy it has also had great impact on techniques and theory in microbial ecology. A study by Ward *et al.*, (1990) of the cyanobacterial mat of Octopus spring, Yellowstone Park which used a method of direct extraction and analysis of 16S rRNA sequences reported that none of these sequences matched those of bacteria which had been recovered and identified using traditional culturing methods. This implies that traditional methods used at this site, and probably many others well studied by these methods, have not given a true picture of their microbial ecology. However 16S rRNA sequencing alone cannot give any information on phenotypes and biogeochemic capabilities of the microbial population.

1.3.4.5 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

If there is variation in the ribosomal RNA sequence between two strains then it is possible to use restriction digestion to differentiate between them, a method known as amplified ribosomal DNA restriction analysis (ARDRA). This technique employs the polymerase chain reaction (PCR) to obtain sufficient ribosomal RNA gene fragments (rDNA) for analysis. This rDNA is then subjected to digestion with a particular restriction enzyme which will cleave the rDNA into a number of chains of differing length whose size can be determined using electrophoresis with appropriate

standards. The pattern of bands produced by the different fragments during electrophoresis can then be compared to those produced by other strains subjected to the same process with the same restriction enzyme. If the restriction enzyme has cut the rDNA of the two different strains at different positions then the different banding patterns can be used to differentiate between them.

This method has been applied to both the 16S and intergenic 16S-23S spacer region with some success for a range of bacteria (Massol-Deya *et al.*, 1995; Vaneechoutte *et al.*, 1992). It has been suggested that it may be useful in analyzing mixed populations (Massol-Deya *et al.*, 1995).

The advantages of this method are that little cellular material is needed for analysis, as few as 1-10 cells can give a satisfactory result (Massol-Deya *et al.*, 1995), it is fast, and it is easy to analyse.

The limitations of this method are imposed by the necessity of choosing of an appropriate restriction enzyme. It cannot be assumed that a restriction enzyme will produce different banding patterns for two strains with different DNA codes - they must differ at the sites for which the enzyme is specific. Theoretically, banding patterns could differ for closely related organisms which vary at one site and show no variation for more distantly related organisms which do not vary at the enzyme specific sites. It is also necessary to choose an enzyme which does not act at too many sites on the rDNA as this can produce a large number of small bands which are difficult to resolve and compare. In some cases an enzyme for the most appropriate site may not be available.

1.3.4.6 Restriction Fragment Length Polymorphism (RFLP)

RFLP is a method which, like ARDRA, uses restriction endonuclease digestion to differentiate between strains. In this method the whole extracted DNA is digested and the bands separated by gel electrophoresis and southern blot. The fragments are then probed with a labelled oligonucleotide, specific for a particular conserved sequence present in all strains. Usually part of the rDNA codon which occurs at several sites on a single bacterial chromosome is chosen (Johansson *et al.*, 1995). In most strains of *Streptomyces* spp. this method will yield a pattern of six bands (Mehling *et al.*, 1995).

1.3.4.7 16S-23S Internal Spacer Polymorphism

In most prokaryotes the genes coding for the 16S and 23S ribosomal fragments are separated by a spacer region. This spacer region can vary greatly in length and sequence between different species and between the multiple rRNA genetic loci often found in a single species (Jensen *et al.*, 1993). These variations may be due in some cases to the differences in the number of and codes for tRNA contained in these spacer regions (Loughney *et al.*, 1982; Brosius *et al.*, 1981). However no tRNA genes appear to be located in this region for any genera of the *Actinomycetales* so far investigated, including members of the genera *Frankia, Streptomyces* and *Bifidobacterium* (Normand *et al.*, 1992; Suzuki *et al.*, 1988; Leblond-Bourget *et al.*, 1996).

It is possible to use these spacer regions to differentiate between different species of prokaryotes (Barry *et al.*, 1991) and in some cases between strains (Hain, T. O., 1996, personal communication). This can be especially useful in some cases where it is otherwise hard to distinguish between strains on the basis of physiological characteristics (Wunschel *et al.*, 1994). This is not always possible however and for some groups the spacer patterns and even sequences of the spacer loci show little or no differentiation between strains (Drebot *et al.*, 1996).

The fastest way of determining the spacer pattern for eubacteria is by PCR amplification of the spacer region followed by electrophoresis of PCR product with appropriate molecular weight standards. Restriction digestion of the PCR product can be used to give a more complex pattern and distinguish between strains not resolved sufficiently by electrophoresis of the primary PCR product (Jensen *et al.*, 1993).

Spacer pattern analysis appears to be a fast and accurate way of identifying many isolates to species and sometimes strain level. It cannot be used as a tool for developing a phylogenetic classification however. Spacer patterns typically comprise one to four fragments of high molecular weight (approx. 250-1500bp) nucleic acid sequences and there is no indication that these patterns are more likely to be similar in size or number of fragments between related species than between un-related species.

Sequencing of the spacer loci can be used for determining phylogenetic relationships between strains which are too closely related to be compared

using the more highly conserved ribosomal code alone as was demonstrated for members of the genera *Bifidobacterium* (Leblond-Bourget *et al.,* 1996). This method may be useful in a similar way for other genera of actinomycetes which do not contain code for tRNA at this loci and are therefore more likely to show greater sequence variation.

1.3.4.8 Denaturing Gradient Gel Electrophoresis (DGGE)

A partially melted DNA molecule will move through polyacrylamide gel at a much slower rate than if it is in a complete helical form. Sequence variation in DNA will cause differences in melting temperature (Muyzer *et al.,* 1993). The DGGE method mimics an increasing temperature by incorporating an increasing gradient of DNA denaturing agents into a polyacrylamide gel and then running multiple DNA samples, differentiating between organisms on the basis of where in the gel the DNA reaches its melting point (Muyzer *et al.,* 1993).

It is possible to analyse whole genomic DNA by this method or to selectively amplify particular sequences with PCR prior to DGGE. Muyzer *et al.,* 1993 reported that they could use this method to identify members of a bacterial community which represented only 1% of the total population. It is possible to apply specific probes to the bands obtained from an environmental sample to determine which groups are present and to cut out DNA bands for sequencing if this is desired. The intensity of a band gives a semi-quantitative measure of the numbers of an organism in the environment, but PCR of the original DNA would mean no idea of proportions could be obtained.

This method has potential for use in microbial ecology as it can both separate genomic material for further study and can be used for a method of identification and profiling of bacterial populations in environmental samples. It is relatively quick and easy to perform (Muyzer *et al.,* 1993). This method could perhaps be used to separate closely related strains and may therefore be useful in a polyphasic approach to taxonomy.

1.3.4.9 Oligonucleotide Probes

If a particular code in the genome is specific to a particular microorganism then it is possible to develop an oligonucleotide probe complimentary to this region. A fluorescent dye attached to the oligonucleotide probe then

allows detection of the microorganism through fluorescence microscopy (DeLong *et al.*, 1989). Probes have been developed in this way for regions which are species and genus specific (Stackebrandt *et al.*, 1991; Stackebrandt and Charfreitag, 1990) on the 16S rRNA molecule. The 16S rRNA molecule is ideal for this purpose because it is sequenced routinely in the description of a species and because rRNA is abundant in bacterial cells. Fluorescent probes for sequences found on rRNA therefore allow easy visualisation (DeLong *et al.*, 1989).

Probes can range in specificity from those which separate major divisions (eukaryotes, eubacteria and archaebacteria) to those which can separate individual species within a genus (DeLong *et al.*, 1989). It is possible to use multiple probes attached to different fluorescent dyes simultaneously and combine this with either fluorescence microscopy or flow cytometry in order to analyse bacterial communities (DeLong *et al.*, 1989).

Certain hypervariable regions of the 16S and 23S rRNA molecule have been recognised as being potentially useful for species specific probes and probes for these areas have been used to develop probes which alone or sometimes in a combination of two or more probes can be used to identify individual species (Stackebrandt *et al.*, 1991; Gobel *et al.*, 1987; Liesack *et al.*, 1990; Stackebrandt and Charfreitag, 1990; Spierings *et al.*, 1992).

A region for the construction of a genus specific probe has been reported for the genus *Streptomyces* and species specific probes for a number of different species of *Streptomyces* (Stackebrandt *et al.*, 1991) and *Actinomyces* (Stackebrandt and Charfreitag, 1990).

Genus specific probes have also been used effectively in ecological studies both to identify members of specific genera, and to determine proportions of bacteria from different genera in a wide variety of environments (Ward *et al.*, 1990; Manz *et al.*, 1993; Holben *et al.*, 1988).

1.3.5 Other Methods of Identification and Classification

1.3.5.1 Serology

Serology can be used compare proteins produced by various strains. Results from the use of an indirect enzyme-linked immunosorbent assay (IND-ELISA) has shown good correlation with clusters determined by numerical taxonomy (Kirby and Rybicki, 1986). IND-ELISA involves coating a plastic well with bacterial antigen, binding of a specific antigen to a specific antibody and then binding a second antibody-specific antibody linked to a substrate-specific enzyme.

The study by Kirby and Rybicki used polyclonal antibodies and used cross reactivity as a measure of similarity between strains.

The advantages of this method are that it is rapid, can be easily mechanised and the amount of each protein can be determined quantitatively.

The disadvantages are that for identification purposes it would always be necessary to have a large library of polyclonal antibodies on hand. Its application in studying the ecology of microorganisms may be limited by cross reactivity of closely related environmental microorganisms.

This method may be useful in a developing systems of classification as it can identify relative similarities in protein compositions between microorganisms as an indirect measure of the genetic code.

1.3.5.2 Phage Typing

Numerous bacteriophages have been isolated which are specific for all genera of actinomycetes except most maduromycetes with a wall type III (Korn-Wendisch and Scheider, 1992). These phages can be useful for identification of actinomycetes to genus and sometimes species level (Korn-Wendisch and Scheider, 1992; Prauser, 1984).

Genus specific phages have indicated that some strains are placed in the wrong genus (Prauser, 1984). These findings have then been confirmed by biochemical and molecular techniques. Phage specific for members of a particular genera therefore contribute to a phylogenetic system of classification .

Species specific phages are useful in only a limited way as few have been found, however a pattern of sensitivities to a range of phages can aid species identification in a polyphasic approach (Korn-Wendisch and Scheider, 1992).

1.3.5.3 Ribosomal Typing

Bacterial ribosomes contain more than 50 species of ribosomal proteins. Ribosomal proteins have a highly conserved structure, more so than most other bacterial proteins (Ochi, 1992). Analysis of variation in patterns of ribosomal proteins by two-dimensional polyacrylamide-gel electrophoresis has been reported to show a strong correlation with other taxonomic data including that from molecular techniques.

Ribosomal typing has the advantage of being quick, relatively cheap and unaffected by either growth phase or medium. Because differences also appear to accord well with phylogenetic relationships it could become a useful method for both developing polyphasic taxonomies and rapid identification (Ochi, 1992).

The amino acid sequence of one of these ribosomal proteins, AT-L30 was determined for 81 species of *Streptomyces* by Ochi (1995). These sequences were compared and the resulting phylogenetic tree was compared to others based on numerical classification and 16S rRNA sequencing. The clusters formed by AT-L30 showed considerable similarity to those resulting from numerical classification (Williams *et al.*, 1983a) but also some significant differences. There was a closer correlation between the AT-L30 and 16S rRNA results, indicating that this method could be useful in resolving bacterial phylogenies and providing supporting data for a polyphasic approach to classification.

1.3.5.4 Amino Acid Sequence Analysis of Proteins.

Amino acid sequences are a direct reflection of the genetic code. They can therefore be used in a similar manner to nucleic acid sequences for comparing phylogenetic relationships between related strains. To be useful in this way a protein must have a wide (if not universal) distribution throughout the strains under comparison, and the degree of variation between the amino acid sequences must be appropriate, i.e. enough to

indicate variation between closely related strains but not so great that the differences overwhelm the similarities.

A protein which has been reported to fulfil this role for the members of the genus *Streptomyces* is subtilisin inhibitor and its homologs, known collectively as *Streptomyces* subtilisin inhibitor-like proteins (SSI-like proteins). Sequences of these proteins have been reported to clearly differentiate between a number of *Streptomyces* species whose relationships based on 16S rRNA sequence data were ambiguous (Taguchi *et al.*, 1995).

As most proteins show greater variation in their sequences over time than do ribosomal RNA nucleic acid sequences, they could be useful in determining otherwise ambiguous phylogenetic relationships. Exceptions to this general rule may occur in the more highly conserved ribosomal proteins (see 3.4.3 above).

1.3.5.5 Pyrolysis Mass Spectrometry

Pyrolysis mass spectrometry compares the differences in the mass spectra of volatile products formed by pyrolysis of bacteria at a temperature 530°C. Comparison of spectra prepared by carefully standardized procedures have been reported to show variation of only 3% in triplicate samples (Sanglier *et al.*, 1992) and are capable of distinguishing between actinomycetes to species level.

Clusters determined by this method correlate well with those determined by biochemical and DNA similarities. Comparing environmental strains with this method indicates that it could be a useful method for eliminating duplicate strains prior to pharmacological assay (Sanglier *et al.*, 1992).

A problem with this method is that of "instrumental drift". Despite standardisation of methods there appears to be a change in profile for strains over time due to changes in the mass spectrometer (Sanglier *et al.*, 1992). This also indicates that there could be problems in comparing profiles between laboratories using different machines.

1.3.6 The Polyphasic Approach

To determine a phylogenetic classification of bacteria which is both true and practical it is necessary to employ a polyphasic approach, employing a combination of molecular, chemical and numerical taxonomic methods (Murray *et al.*, 1990). This approach attempts to base a system of classification on a reconciliation of differences in the genetic and phenetic approaches (Embley and Stackebrandt, 1994).

For a large group of closely related strains such as members of the genus *Streptomyces* a polyphasic study is a large task. Some clusters defined by the numerical taxonomic study of Williams *et al.*, (1983a) have been supported by chemotaxonomic and molecular taxonomic studies (Saddler *et al.*, 1987; Mordorski *et al.*, 1984; Witt and Stackebrandt, 1990; Labeda and Lyons, 1991). However, some other clusters determined in the Williams *et al.*, (1983a) study have been shown by DNA-DNA hybridization studies to show as great an amount of variation between members within particular clusters as there is between members of different clusters (Witt and Stackebrandt, 1990; Labeda and Lyons, 1991). DNA:DNA hybridization is not accurate in determining phylogenetic depth of clusters (Witt and Stackebrandt, 1990) so these results may indicate that some of the clusters are deeply rooted rather than made up of unrelated members.

A number of studies have used a polyphasic approach to classify non-streptomycete Actinomycete taxa. These are shown in Table 1.9. These reports highlighted the confused nature of previous systems of classification and the necessity for using a variety of methods in order to determine which are the most useful for re-defining taxa.

Under some circumstances a polyphasic approach can give conflicting results (Rainey *et al.*, 1995). Biases are introduced into the analysis by the selection of organisms for comparison. The solution to this problem may be, if possible, to increase the number of closely related organisms under comparison.

Table 1.9: Studies using polyphasic techniques for classification and description of actinomycete genera.

Genera studied	Taxonomic techniques employed	References
Actinoplanes and related genera	Colour of substrate mycelium, formation of sporangia, pigment production, antimicrobial activity, biochemical tests, substrate utilization and degradation, tolerance to various growth conditions, fatty acids, menaquinones	Goodfellow *et al.*, 1990
Actinoplanes, *Ampullariella* and *Amorphosporangium*	Whole cell sugars, phospholipids, menaquinones, peptidoglycan type, spore morphology and motility, DNA-DNA hybridization, 16S rRNA oligonucleotide catalogueing	Stackebrandt and Kroppenstedt, 1987
Faenia and *Saccharopolyspora*	Mycelial pigmentation and morphology, spore morphology, peptidoglycan type, whole cell sugars, mycolic acids, phospholipids, fatty acids, menaquinones G+Cmol%, phage typing, enzyme and protien patterns	Korn-Wendisch *et al.*, 1989
Thermophilic Actinomycetes	Sugar type, fatty acids, menaquinones, spore chain morphology, spore surface morphology, aerial mycelium pigmentation, phage sensitivity	Greiner-Mai *et al.*, 1987
Nocardiopsis	Whole cell sugars, menaquinones, phopholipid type, fatty acids, morphology, substrate utilization and degradation, salt tolerance and lysozyme resistance	Grund and Kroppenstedt, 1990
Actinomadura and *Microtetraspora*	G+Cmol%, fatty acids, menaquinones, polar lipids and sugars, 16S rRNA sequence	Kroppenstedt *et al.*, 1990
Actinomadura and*Nocardiopsis*	DNA-DNA hybridization, DNA-rRNA hybridization, menaquinones, whole cell sugars, fatty acids	Fischer *et al.*, 1983
Mycolateless wall chemotype IV actinomycetes	Menaquinones, phospholipids, fatty acids, spore production, mycelium morphology	Kothe, *et al.*, 1989
Actinomadura, *Microbiospora*, *Microtetraspora*, *Micropolyspora* and *Nocardiopsis*.	DNA:DNA hybridisation, fatty acids, menaquinones	Poschner *et al.*, 1985

There is disagreement on what the outcome of on-going classification will be. Some authors have proposed that the actinomycetes, and genus *Streptomyces* in particular, are "over-speciated" (Williams *et al.*, 1983a; Kampfer *et al.*, 1991; Bull *et al.*, 1992) while others say they are "under speciated" (Goodfellow, *et al.*, 1992; Labeda, 1992). New genera are continually discovered and described (e.g. *Thermocrispum,* was nominated as a new genus on the basis of a distinctive chemotaxonomy and 16S rRNA sequence [Korn-Wendisch *et al.*, 1995]), and many new species are also being discovered. At the same time, previously described species and genera are being nominated as synonyms (Korn-Wendisch *et al.*, 1989; Henssen *et al.*, 1987; Grund and Kroppenstedt, 1989; Kroppenstedt *et al.*, 1990). Actinomycete taxonomy is still under development and more taxonomic information needs to be collected in all fields in order to develop a phylogenetic system of classification with confidence.

Chapter 2: Materials And Methods

2.1 Collection and Storage of Samples

A 5 mL sterile plastic tube was opened within 10 cm of the sample site. The tube was pushed into the soil and pulled out with a plug of soil inside and immediately re-capped. These tubes were stored at -70°C for a period of up to three months before isolation of actinomycetes was attempted. As Antarctic soil organisms must be tolerant to freeze thaw cycles, freezing was considered the best method for sample preservation. All samples were collected during the Antarctic summer of 1992/93. Descriptions of collection sites are given in Table 2.1 .

2.2 Isolation

2.2.1 Micromanipulation

A Skerman Micromanipulator was used to isolate hyphal actinomycetes using a method modified from Skerman (1968), outlined as follows.

2.2.1.1 Preparation and Inoculation of Media

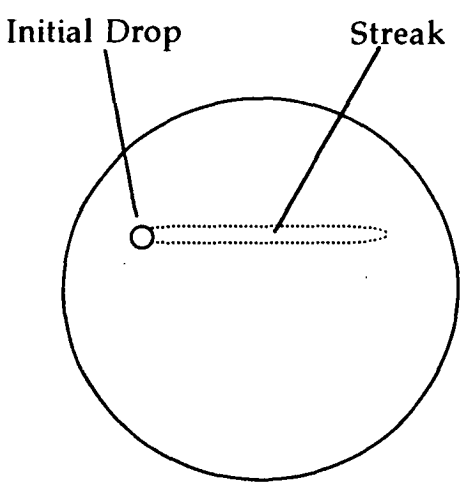
Plates of Micromanipulation or Seawater Micromanipulation Agar (Appendix 1) were prepared by pouring sterile medium into petri dishes which had been laid on a completely flat, horizontal surface in a laminar flow cabinet. Seawater Micromanipulation Agar was used as some of the soils collected in the present study were highly saline and may have contained bacteria favouring or requiring saline conditions for growth.

Table 2.1 Description of Antarctic sites from which soil was collected for attempted actinomycete isolation.

Sample No.	Date	Area	Site description	Soil Description	Eukaryotic Life
1	20/11/92	Davis Base, Vestfold Hills	Rocky valley, Ace Lake, southern shore, beneath a black rock	Rocky	None observed
2	20/11/92	Davis Base, Vestfold Hills	Salty Basin, shore of unfrozen lake south of Ace Lake, rock free.	Thin orange layer over black, moist, aerated	Two penguins nearby. Feather next to sample area.
3	20/11/92	Davis Base, Vestfold Hills	South west slope of Ace lake, approx. 15m above shoreline	Aerated and moist	None observed
4	20/11/92	Davis Base, Vestfold Hills	Beneath quartz rock in same area as sample number 3	as for number 3	Green algae scraped from beneath rock
5	20/11/92	Davis Base, Vestfold Hills	Beneath quartz rock in same area as sample number 3	as for number 3	Green algae scraped from beneath rock
6	23/11/92	Davis Base, Vestfold Hills	Beneath quartz rock near Deep Lake weather station. Southerly aspect.	moist, coarse	Occasional humans and birds (non-penguin)
7	23/11/92	Davis Base, Vestfold Hills	North shore of Organic Lake.	moist	Penguins common
8	23/11/92	Davis Base, Vestfold Hills	Exposed ground halfway between Organic Lake and ocean, near small pond	moist	Penguins common
9	23/11/92	Davis Base, Vestfold Hills	North shore of Burton Lake.		Black lettuce lichen nearby
10	23/11/92	Davis Base, Vestfold Hills	Ridge on north-west shore of Pendant Lake	moist	None observed
11	30/11/92	Davis Base, Vestfold Hills	Magnetic Island, in penguin colony, open ground with snow melt	moist	Penguins very common
12	30/11/92	Davis Base, Vestfold Hills	Magnetic Island, near penguin colony.	dry	Penguins nearby
13	30/11/92	Davis Base, Vestfold Hills	Near pond of melt-water, Magnetic Island, below penguin colony.		Penguins nearby
14	4/12/92	Davis Base, Vestfold Hills	At bottom of penguin rookery	ornithogenic	Penguins very common
15	4/12/92	Davis Base, Vestfold Hills	Beach near penguin rookery		Penguins common
16	4/12/92	Davis Base, Vestfold Hills	Moss and lichen beds		Dried out moss and lichen bed, observed green in Jan. 1988
17	7/12/92	Davis Base, Vestfold Hills	Trajer ridge moss and lichen bed, near fresh water lakes.		Moss and lichen bed
18	7/12/92	Davis Base, Vestfold Hills	As for site 17, same moss/ lichen bed.		Moss and lichen bed
19	7/12/92	Law Base, Mirror Peninsula	Disturbed area 500m north-north-east of base.		Vehicle and foot tracks.
20	7/12/92	Law Base, Mirror Peninsula			None observed
21	7/12/92	Law Base, Mirror Peninsula			None observed
22	7/12/92	Davis Base, Vestfold Hills	North shore of Clear Lake	moist	Well developed moss and lichen communities.
23	7/12/92	Davis Base, Vestfold Hills	Flat north shore of small lake west of Lake Fletcher.	dry	Algal mat, a few penguins
24	7/12/92	Davis Base, Vestfold Hills	Shore of small very salty pond west of base		None observed

One mL of sterile water was pipetted into a clean sterile tube and 0.1 g of a soil sample was added. The tube was capped and vortexed for 20 seconds. The tubes were placed in a 50°C incubator for 10 minutes as a method for reducing numbers of non-Actinomycetes (Labeda and Shearer, 1990). The tubes were again vortexed for 20 seconds and 25 µL was immediately removed with a sterile pipette. A drop was placed on one of the prepared plates in the position shown in Figure 2.1. The plate was immediately tilted to allow the drop to flow across the plate in an even streak as shown in figure 2.1. This procedure was repeated for each soil without the heat-treatment stage during which each sample was exposed to room temperature for approximately ten minutes. Each plate was then placed in an incubator at 10°C and the inoculation streak was examined weekly under a phase-contrast microscope using X32 long working distance lens for microcolonies showing morphology typical of hyphal forming actinomycetes. This procedure was performed for every sample on both Micromanipulation Agar and Seawater Micromanipulation Agar.

Figure 2.1 Method for inoculation of plate in order to isolate bacteria using the Skerman micromanipulator



2.2.1.2 Construction of Tools for Micromanipulation

Glass tools for micromanipulation were constructed using a microforge (Skerman, 1968). Briefly, a glass pasteur pipette was heated until the glass was soft and drawn out into a thin capillary. A section of the capillary was placed into a capillary holding carriage and the carriage was attached to a microscope such that the tip of the capillary tube was in focus in the viewing field of a X4 lens. A microforge, consisting of a thin wire was

heated to a dull red colour. The base of the wire was pressed against the end of the tube, and when the glass was sufficiently fluid it was drawn out into a thinner capillary. The current through the microforge was reduced to a temperature at which glass could be moulded but not melted. The microforge was then used to gently push the thin capillary into the shape of a hook.

2.2.1.3 Isolation of Strains by Micromanipulation

The glass hook tool in the capillary carriage was attached to a long working distance X32 lens (Leica, L32, Phase Contrast) so that the hook was immediately above the focal depth of the lens. An agar plate which had been inoculated as above (section 2.2.1.2) was placed on the microscope platform and the platform raised so that microcolonies at one end of the inoculation streak would come into focus. The platform was moved slowly so that the field of view moved along the length of the inoculation streak and microcolonies could be viewed. When a bacterial colony showing hyphal morphology was observed the microscope stage was lowered and the glass hook was moved down into focus. The platform was now raised gently until the hyphal microcolony was again in focus at which stage the hook was pressing into the colony. The platform was now moved gently back and forth so that the hook was run several times through the colony, and then the platform was lowered. The hook was examined for the presence of hyphae: if none were present the platform was raised and the procedure was repeated until hyphae were on the hook. The platform was then moved until a clean part of the agar plate was below the hook. The hook was then lowered onto the agar and moved back and forth until the hyphal material came away. The hook was used to sufficiently score a square in the agar surrounding the deposited hyphae, so as to locate the site with the naked eye, and an area next to the site was scored with a roman numeral to serve as a label for the site.

Plates so treated were placed back into the 10°C incubator and the isolated hyphae were examined daily. When a micro-colony developed at a site to which hyphae had been removed by micromanipulation, the section of agar on which it was growing was removed with a sterile scalpel and used to inoculate a fresh plate of sporulation agar (Appendix 1). Once colonies were observed on the sporulation agar a slope of sporulation agar was inoculated and stored at 4°C.

2.2.2 Nomenclature of Antarctic Strains

In this report "Isolate" refers to bacteria isolated from an Antarctic soil. Each isolate is referred to by a number, which corresponds to the soil number from which it was isolated, and a letter, which was assigned to each isolate in the order in which it was removed from the inoculation streak. The name of isolates taken from salt-water micromanipulation agar is preceded by the letter "S".

2.3 Storage And Maintenance of Cultures

Isolates were stored on agar slopes for periods of up to three months but as Actinomycetes tend to lose some of their secondary characteristics, such secondary metabolite and pigment production, after sub-culturing (Hirsch *et al.*, 1985; Srinivasan *et al.*, 1991; Lechevalier and Lechevalier, 1985), the original isolates were freeze-dried (Appendix 4).

2.4 Soil Analysis

2.4.1 pH

The pH of the soil was determined using a method from Loveday, 1974. One gram of soil sample was placed in a clean test tube. The tube was left in an oven at 105°C for twenty four hours to allow the soil to dry. The sample was sieved through a 2mm steel mesh. Any remaining soil was ground in a mortar and re-sieved until all the soil could fit through the mesh, then 0.4g of soil was put into a clean test tube, 2mL of freshly distilled water was added and the tube was capped, vortex mixed for 1 minute and allowed to stand overnight. The following day the sample was again agitated for 1 minute on the vortex mixer, allowed to settle for 30 minutes, and the pH measured.

2.4.2 Conductivity

The conductivity of the soil samples was determined using a method derived from Loveday, 1974. The soil was air dried, sieved and collected as for pH above. A 1 mL packed volume of the soil was placed in a clean bottle and 2.5 mL of saturated calcium sulphate solution was added. The mixture was shaken for 15 minutes at approximately 275 strokes/min. at 20°C and then filtered through a Whatman No. 2 filter paper. The filtrate was

collected in a clean bottle and retained. 2.5mL of 0.02M KCl was placed in a 20°C water bath and allowed to stand for 20 minutes. The KCl solution was removed and its conductivity measured with a conductivity meter (Radiometer Copenhagen, CDM 3). The cell constant value was determined by the following formula:

$$\text{Cell constant} = \frac{2501}{\text{conductivity of 0.02KCl at 20°C}}$$

The conductivity of 0.02KCl at 20°C should be 2501×10^{-6}. Any minor variations from this measure were used to standardise conductivity readings from test samples

The filtrate was placed in the water bath for 20 minutes and then its conductivity measured. The soil conductivity was determined using the following formula:

$$\text{Soil conductivity} = \text{filtrate conductivity} \times \text{cell constant}$$

Determining laboratory density of soil

Twenty mL of soil which had been ground using a clean mortar and pestle and sieved with a 2mm wire mesh was weighed and the result divided by 20. The resulting number was the "laboratory density" of the soil.

The original method stated that if the laboratory density is outside the range 0.98-1.02 the conductivity can be calculated using the formula:

$$\text{C corr.} = \frac{58.4}{(C-2000) \times (103.4 - 45D) + 2000}$$

C.corr. = corrected conductivity
C = conductivity obtained from filtrate x cell constant
D = laboratory density

This was not necessary for any of the measurements taken in this study which all fell within the 0.98 - 1.02 range.

2.4.3 Water content

A crucible was placed in an oven at 105°C, then transferred into a desiccation chamber and allowed to cool to room temperature. The crucible was weighed and a soil sample was placed in the crucible which was weighed again. The crucible was then placed in the 105°C oven for a further 48 hours. The crucible was transferred to a desiccation chamber and weighed. The percentage water of the soil was calculated according to the following formula:

$$\%\text{water of soil, by weight} = \frac{(\text{wt of crucible + soil after drying}) - \text{wt of crucible}}{(\text{wt of crucible + soil before drying}) - \text{wt of crucible}} \times 100$$

2.4.4 % Loss on Ignition

The crucible containing the dried soil which had been used to determine water content (above) was placed into a furnace . The furnace was heated to 600°C and the crucible was left at this temperature overnight. The following day the furnace was allowed to cool to 100°C, the crucible was then transferred to a desiccation chamber and allowed to cool to room temperature before being weighed. The percentage loss on ignition (LOI) was calculated using the following formula:

$$\text{LOI} = \frac{(\text{wt of crucible + soil after ignition}) - \text{wt of crucible}}{(\text{wt of crucible + soil before ignition}) - \text{wt of crucible}} \times 100$$

2.5 Morphology

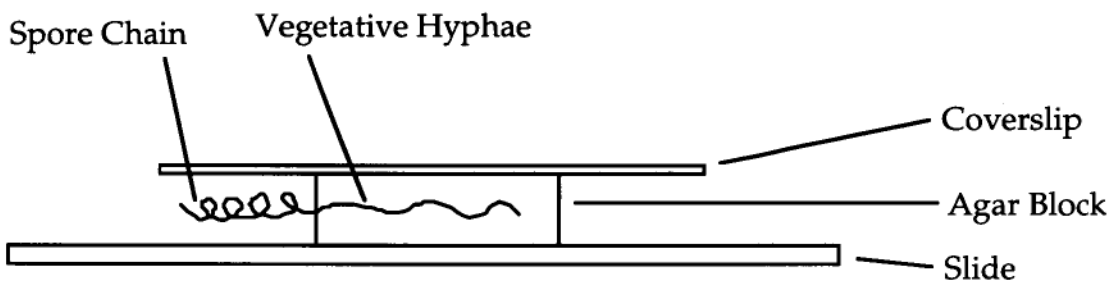
2.5.1 Microscopy of Spore Chains

Spore chain morphology was determined using a method modified from Cross (1989).

Sterile microscope slides were placed in a sterile moist chamber. A thin plate of Modified Sporulation Agar (Appendix 1) was prepared and cut into small blocks. Each of these blocks of medium was then transferred to one of the sterile slides in the moist chamber using a sterile scalpel blade. Each block was inoculated with mycelia taken from a freshly grown plate of one of the soil isolates. A sterile cover slip was then placed over each inoculated block (see Fig. 2.2) and the sealed moist chamber was placed in a 25°C incubator for one week. After a week, the moist chamber was removed and

each slide was examined under a phase contrast microscope at X400 magnification. If no aerial mycelia were visible the slide was incubated for a further three days after which the slide was examined again. If after 10 days no aerial mycelia had appeared on a slide the whole process was repeated for that particular isolate. Spore chains on the aerial mycelia were identified as reflexibles (straight or wavy), retinaculiaperti (hooked or looped) or spirales (tight spirals) (Locci, 1989).

Figure 2.2: Cut-away section through slide preparation for viewing Aerial hyphae in sporulating actinomycetes.



2.6 Antibiotic Screening

Each isolate was screened for antibiotic production against four test organisms, *Bacillus subtilis* (Sydney University, Strain 21-6 [Whitehouse]) *Pseudomonas aeruginosa* (ATCC 27853) *Staphylococcus aureus* (ATCC 660) and *Escherichea coli* (Melbourne University, Strain 200).

Plates of modified sporulation agar (Appendix 1) were prepared. Each plate was inoculated with four different soil isolates, each by a single touch with an inoculation loop at one of four separate quarters of the plate. The inoculated plates were incubated at 25°C for four days after which time growth of actinomycete colonies were observed in all cases. After four days the plates were removed and, using a sterile spatula, the agar was inverted. The newly exposed surface of the agar was inoculated with 100 µL of one of the four test organisms listed above which had been grown to maximum growth in nutrient broth. The inoculum was spread evenly over the plate with a sterile glass hockey stick. The plates were then incubated at 25°C until a lawn of bacteria had grown over the plate. Zones of inhibition were observed and measured.

2.7 Physiology

2.7.1 Carbohydrate Utilization

A sterile 4x6 well microtitre plate was used to determine the ability of each isolate to utilize each of 20 compounds as a sole carbon source, recommended for differentiation between *Streptomyces* species in Bergey's Manual (Locci, 1989). The carbon sources used were adonitol, arabinose, cellobiose, dextran, fructose, galactose, glucose, meso-inositol, inulin, lactose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, salicin, sucrose, trehalose and xylose.

2.7.1.1 Agar Preparation

A high and a low salt solution (Appendix 1) were prepared, sterilized for 20 minutes at 121°C and placed into a 60°C water bath. A 10% carbon source solution was prepared for each of the 20 sugars used and sterilized by filtration. One mL of each sterile filtered carbon source was pipetted into a separate sterile 20 mL McCartney bottle. The bottles were then placed in a 60°C water bath and left for 30 minutes to ensure the solutions were at 60°C throughout. The carbon source solutions were removed one at a time and, working in a sterile laminar flow cabinet, 5 mL of both the high and low salt solution were added to each McCartney bottle which was mixed by up-ending it 3-4 times and immediately placed back in the 60°C water bath. When all 20 flasks had been treated in this way they were removed from the water bath one at a time and 1 mL aliquots of each solution were pipetted each into a single well in each of twenty plates using the following pattern:

Adonitol	L-arabinose	Cellobiose	Dextran	D-fructose	
D-galactose	D-glucose		Inulin	D-lactose	
Mannitol	D-mannose	D-melezitose	D-melibiose	meso-Inositol	Raffinose
L-rhamnose	Salicin	Sucrose	Trehalose	D-xylose	Negative Control

The plates were allowed to set and stored at 4°C pending inoculation.

2.7.1.2 Agar Inoculation and Analysis

Cultures were grown on sporulation agar (Appendix 1) for 2-3 weeks. Twenty mL of sterile distilled water were poured on the plate which was then scraped vigorously with a sterile metal strip. The suspension was poured into a sterile plastic box and swirled to allow even distribution of the suspension. A sterile inoculation tool made from a metal plate pierced with 23 metal pins was used to inoculate the 21 test wells simultaneously by dipping it in the bacterial suspension and then placing lightly on the test plate such that each of the pins entered a single well. The plate was then sealed and placed in a 25°C incubator. After five weeks the plate was removed and each well was rated as -, +, ++ or +++ based on the amount of growth when compared with growth on the control medium which lacked an organic carbon source.

2.7.2 Melanin Production

Each of the isolates was streaked out onto a plate of Peptone-Yeast Extract-Iron Agar (Appendix 1). After four weeks the plates were examined for melanin production which was indicated by dark brown pigment surrounding the colonies (Shirling and Gottlieb, 1966).

2.7.3 Numerical Taxonomy

Characters of each isolate including spore mass colour, spore chain morphology, melanin production, sugar utilization and antibiotic production against test organisms were compared with one another using the program TAXON (Ross and Shields, 1993). Each character was given equal weight. Characters with more than two possible states, such as spore chain morphology and spore mass colour, were coded as multistate variables in order to avoid giving these characters unequal weighting. Comparison was by Burr's strategy (Ross and Shields, 1993).

2.8 Chemotaxonomy

2.8.1 Fatty Acids

Method 1.

2.8.1.1 Cultivation

Isolates were maintained on slopes of Sporulation medium (Appendix 1). A loopful of aerial mycelia was used to inoculate 250 mL of Trypticase Soy Broth (Appendix 1) in a 500 mL flask. The cultures were incubated at 25°C in an orbital incubator-shaker at 200 revolutions per minute. Cultures were microscopically checked for purity at maximum growth, harvested by centrifugation, washed three times in distilled water, (10 000 rpm) and freeze dried.

2.8.1.2 Extraction

Fatty acids were extracted using a modified procedure described by Kroppenstedt (1996). Prior to extraction all glassware was rinsed with Hexane:Methyl-t-Butyl Ether (4:1) to remove possible contaminants. Ten to 15 mg dry weight of cells were placed in a cleaned glass tube with a Teflon-

lined screw top . One mL of saponification agent, NaOH:methanol:de-ionised distilled water (45g:150 mL:150 mL), was added and the Teflon-lined cap screwed on tightly. The mixture was vortex mixed for 30 seconds, incubated in a 100°C water bath for five minutes, checked to ensure no leakage had occurred and placed back in the water bath for a further 25 minutes. The tube was then cooled to room temperature and two mL of distilled water were added. Three mL of extraction solvent, hexane:methyl-t-butyl ether (4:1) and the mixture was vortexed for 30 seconds. After separation of the solvent phases, the top layer, containing non-saponifiable lipids was removed to a round bottomed flask. This extraction step was repeated three times. The solvent was removed from the non-saponifiable lipid fraction with a rotor evaporator, and the remaining lipid dissolved in one mL of chloroform and stored in a gas chromatography vial at -4°C. One mL of 2M H_2SO_4 was added to the solution remaining in the tube, which was agitated with a vortex mixer for 30 seconds, and the pH was checked with indicator paper to ensure a pH of <2 had been obtained. The fatty acids were extracted with 4 mL of extraction solvent using the same procedure as for the non-saponifiable lipid above. The pooled solvent extract was dried by rotor evaporator action and the fatty acid residue was dissolved in one mL of Methylation reagent, chloroform:HCl:methanol (1:1:10), which was placed in a fresh tube and sealed tightly with a teflon lined cap. This tube was agitated for 30 seconds with a vortex mixer and incubated for five minutes in an 80°C water bath. The tube was checked to ensure no leakage had occurred and placed back in the water bath for two hours. After cooling to room temperature, the fatty acid methyl esters (FAMEs) were extracted with one mL of extraction solvent using the same procedure as for the neutral lipids. The pooled solvent extract was removed from the FAMEs by rotor evaporator action, and the FAME residue was dissolved in one mL of chloroform and stored in a gas chromatography vial at -20°C.

2.8.1.3 Analysis

Analysis was performed using a Hewlett Packard 5890 Gas chromatograph. The column was a 50 m x 0.32 mm i. d. cross-linked methyl silicone fused-silica capillary column and a flame ionization detector. Gas chromatography-mass spectrometry was performed on an HP5890 GC and 5970 Mass selective detector. Data were collected and processed on an HP59970C work station.

2.8.1.4 Statistics

The differences in proportion of each fatty acid were compared amongst all isolates using the SAS program (SAS Institute Inc., Cary, NC, U.S.A.). Comparison was by the "average" method, with un-standardized data.

Method 2.

This method was provided by R. M. Kroppenstedt (1996), developed by Prof. Myron Sasser in cooperation with Hewlett-Packard.

2.8.1.5 Cultivation

Isolates were maintained on slopes of Streptomyces Medium (Appendix 1). A loopful of aerial mycelia was used to inoculate 5 mL of Streptomyces Broth (Appendix 1) in a 20 mL test tube with a cap. The cultures were incubated at 25°C in a shaking orbital incubator at 120 revolutions per minute. Cultures were checked for purity at maximum growth, harvested by centrifugation and washed three times in distilled water.

2.8.1.6 Extraction

The washed cell material was transferred to a clean dry 13mm x 100mm screw cap test tube. One mL of saponification reagent (Appendix 1) was added and the tube sealed tightly with a Teflon-lined cap. The tube was vortex mixed for 5-10 seconds and placed in a 100°C water bath. After five minutes the tube was removed and checked for leakage, it was then vortex mixed for 5-10 seconds and returned to the 100°C water bath for a further 25 minutes. The tube was removed from the water bath and allowed to cool to room temperature. Two mL of methylation reagent (Appendix 1) was added, the tube was vortex mixed for 5-10 seconds and heated in a 80°C water bath for 10 minutes after which it was removed and cooled rapidly to room temperature by placing it in a 20°C water bath. The tube was removed from the bath and 1.25 mL of extraction reagent (Appendix 1) was added. The tube was sealed tightly and placed in a rotator which mixed the contents for 10 minutes. The tube was then uncapped and the lower (aqueous) phase was removed with a pasteur pipette and discarded. Three mL of base wash reagent (Appendix 1) was added to the remaining solvent, the tube was capped and placed in a rotator which mixed the contents for 5 minutes. The tube was removed from the rotator and centrifuged for three minutes at

2000 rpm. The upper solvent layer was transferred to a clean gas chromatography vial with a pasteur pipette. The bottle was placed in a rack for analysis.

2.8.1.7 Analysis

Fatty acids were analysis was similar to 2.8.1.3 above, performed with a Hewlett Packard 5898 A Gas chromatograph. Peaks were identified by comparing retention time to that of a set of laboratory standards.

2.8.2 Menaquinones

Menaquinone analysis was based on a method by Tamaoka *et al.,* (1983).

2.8.2.1 Cultivation

Isolates were maintained on slopes of Sporulation Agar (Appendix 1). A loopful of aerial mycelia was used to inoculate 250 mL of Nutrient Broth No. 2 (Oxoid) in a 500 mL conical flask. The cultures were incubated at 25°C in an orbital incubator-shaker at 200 revolutions per minute. Cultures were checked for purity at maximum growth, harvested by centrifugation (10 000 rpm), washed three times in distilled water, and freeze dried.

2.8.2.2 Extraction

Approximately 100mg of freeze dried cells were placed in a tube with a Teflon screw cap. The tube was wrapped in aluminium foil as isoprenoid quinones are susceptible to photo-oxidation Two mL of methanol and 1 mL of hexane were added to the tube and the head space was filled with nitrogen gas before recapping with the teflon lined cap. The tube was shaken for 30 minutes on a wrist action shaker then put into an ice bath and left until the two phases separated. The top layer, hexane, was removed to a clean tube, and 2 mL of ice cold hexane was added to the remaining aqueous layer. The hexane:methanol mixture was shaken for five minutes on a wrist action shaker and then 4-5 drops of sterile 0.3% (w/v) NaCl were added. The tube was then placed on ice and the phases allowed to separate. The top hexane layer was removed and combined with the first hexane fraction. The remaining mixture was discarded. The hexane was reduced to approx. 0.5 mL by evaporation under a stream of nitrogen gas, then the tube was sealed and stored at -20°C.

2.8.2.3 Purification

Isoprenoid quinones were purified by passing them through a column of silica gel. The column was washed with diethyl ether: hexane (1:9) and the extracted isoprenoid quinones in hexane were applied to the column. A mixture of diethyl ether: hexane (1:9) was used to elute the menaquinone fraction which could be seen as a faint yellow band in the column. The menaquinone fraction was collected and the solvent was evaporated under a stream of nitrogen. Immediately upon drying the bottle was filled with nitrogen gas, sealed, wrapped in aluminium foil and stored at -20°C.

2.8.2.4 Analysis by HPLC

Menaquinone samples were analysed by HPLC using a Waters U6K with a Waters 600E systems controller and a Waters Nova-Pak C_{18} 3.9 x 150mm column. The menaquinone was dissolved in 0.2 mL of methanol:di-isopropyl ether (14:86, v/v) from which 0.1 mL was injected into the injection loop. The Peaks were eluted with methanol:di-isopropyl ether (14:86, v/v) at 2 mL/min at approximately 21°C, column pressure was in the range 1630-1690 psi. Menaquinones were detected with an Waters 486 tunable absorbance detector set at 270nm. Elution time and peak areas were recorded using Maxima 820 chromatography work station software on a Samsung computer. Peaks were identified by comparison with standard curves which were constructed from the adjusted elution times of standard menaquinones from organisms with known menaquinone profiles (Appendix 2).

2.9 Molecular Taxonomy

2.9.1 16S rDNA Sequencing

Sequencing of 16SrDNA was performed using the following protocol:

- Culturing of bacteria
- Extraction of DNA
- Purification of DNA by Prep-a-gene
- Polymerase Chain Reaction (PCR) cloning of 16SrDNA segment
- Purification of PCR product by Prep-a-gene
- Gel electrophoresis of PCR product to determine concentration
- Sequencing reaction
- Purification of sequencing reaction product
- Separation of sequencing reaction product by gel electrophoresis

Each of these methods is detailed below.

2.9.1.1. Culturing of Isolates and DNA Extraction (Ward, 1995)

Cells were grown on Glucose-Yeast Extract medium for two weeks at 24°C. They were harvested by scraping with a sterile loop and resuspended in 400 µL saline-EDTA in a sterile 1 mL microfuge tube. 10 µL lysozyme (10mg/mL, Boehringer Mannheim) was added, vortex mixed for one minute, and incubated at 37°C for 30 minutes. 5 µL of 1% (w/v) proteinase K (Boehringer/Mannheim) and 10 µL of 25% (w/v) sodium dodecylsulphate (SDS) were added, the mixture was vortex mixed for 1 minute, and incubated at 55°C for 30 minutes. The tube was removed, centrifuged for 5 minutes at 13000 rpm (Eppendorf benchtop centrifuge 5415C), and the supernatant was transferred to a fresh sterile microfuge tube. 400 µL Phenol (Roti-phenol, Roth) was added, the mixture was vortex mixed for 1 minute, and then centrifuged for 10 mins at 13000 rpm. The top layer was transferred to a fresh sterile microfuge tube, 400 µL Chloroform (J. T. Baker) was added, the mixture was vortex mixed for 1 minute, and then centrifuged for 10 mins at 13000 rpm. The top layer was transferred to a fresh sterile microfuge tube and stored at 4°C.

2.9.1.2. Purification of DNA by "Prep-a-Gene"

This method of DNA purification was conducted using reagents and procedure from the Bio-Rad "Prep-a-Gene" DNA Purification Kit as modified by Rainey *et al.,* (1992). A microfuge tube of extracted DNA extract or PCR amplified DNA stored at 4°C was allowed to come to room temperature. The tube was filled with binding buffer (Appendix 1) and vortex mixed for 1 minute . 10ul of matrix (Bio rad prep-a-gene Matrix) was added and the tube was left to stand at room temperature for 10 minutes. The tube was then vortex mixed and centrifuged at 14000 rpm (Eppendorf benchtop centrifuge 5415C) for 1 minute. The supernatant was decanted, 700 µL of binding buffer was added, the tube was vortex mixed for 1 minute and then centrifuged at 14000 rpm for 1 minute. The supernatant was decanted, 750 µL of washing buffer (Appendix 1) was added, the tube was vortex mixed for 1 minute and then centrifuged at 14000 rpm for 1 minute. The supernatant was decanted, 750 µL of washing buffer added, the tube vortex mixed for 1 minute and then centrifuged at 14000 rpm for 3 minutes. The supernatant was decanted and the tube was centrifuged again for 1 minute and a sterile pipette was used to remove the last of the buffer. 50 µL of water was added to the tube, it was vortex mixed for one minute and left to stand in a 37°C water bath for 15-20 minutes. The tube was removed from the water bath, centrifuged at 14000rpm for 2 minutes, the supernatant, containing purified DNA was removed to a fresh microfuge tube and the remaining tube and matrix was discarded. The purified DNA was stored at 4°C.

2.9.1.3. Polymerase Chain Reaction (PCR)

Reagents were added to a sterile 0.6 mL microfuge tube in the following order:

10X incubation buffer (Boehringer)	10 µL
1mM dNTPs (Boehringer)	20 µL
5'primer (10-30) 0.5 g/l*	1 µL
3'primer (1500) 0.5 g/l*	1 µL
template DNA†	1 µL
Water	66.6 µL
Total volume	99.6 µL

*see Table 2.2

† The template DNA was prepared and purified as in sections 9.1.1 and 9.1.2. It was centrifuged for 1 minute at 14000 rpm immediately before use to ensure no Matrix was present in the reaction mixture.

Table 2.2: Primers used for the amplification of 16S rDNA. These two primers were used as they gave thorough coverage of the three hyper-variable regions in the 16S rDNA of the genus *Streptomyces* (Stackebrandt *et al.*, 1991).

Primer	Binding region*	Primer Sequence (5' to 3')
10-30 forward	10-30	GAGTTTGATCCTGGCTCAG
1500 reverse	1520-1540	AGAAAGGAGGTGATCCAGCC

* Number is based on the *E. coli* numbering system from Brosius *et. al.*, 1981

One tube with distilled water added in place of the template DNA and one tube containing pure DNA from a previous successful extraction were always included in a batch to serve as negative and positive controls respectively.

The mixture was vortex mixed for 1 minute and centrifuged (Eppendorf benchtop centrifuge 5415C) at 14000 rpm for 1 minute, then overlayed with 80 µL of sterile light white mineral oil (Sigma) and placed in the thermal cycler (Perkin Elmer DNA Thermal Cycler 480). The tube was heated to 98°C for three minutes and 0.4 µL *Taq* polymerase 5U/µL (Boehringer) was added

below the oil overlay. The thermal cycler was then run through the following profile:

28 cycles:	52°C	1 min.
	72°C	2 min.
	83°C	1 min.
final cycle:	52°C	1 min.
	72°C	5 min.

cool to 4°C

The final product was stored at 4°C prior to purification by the Prep-a-gene protocol (section 2.9.1.2).

2.9.1.4. Gel Electrophoresis of PCR Product to Determine Concentration

NB: Gloves were worn throughout this procedure and whenever handling the gels as ethidium bromide is Toxigenic.

The following were added to a 250 mL flask:

Distilled water	90 mL
X10 TBE (Appendix 1)	10 mL
Agarose (MetaPhor)	1 g

The flask was heated until the agarose was melted and allowed to cool until it was just comfortable to touch before adding 3 µL of ethidium bromide (Boehringer) which was stirred in thoroughly, taking care to prevent the formation of bubbles. The contents of the flask were then poured into the gel mould, well-combs providing sufficient wells for the number of samples to be run were put in place, and the gel was allowed to set. The gel was placed in the electrophoresis apparatus (Gibco Br1 Horizon) and TBE 1X buffer (Appendix 1) was added so as to cover the gel. The combs were removed and the samples prepared in a microwell plate, mixing 3 µL of loading buffer with 5 µL of PCR product or 2 µL of the standard (Molecular weight marker VIII Boehringer Mannheim). Each sample was then pipetted into its own well in the gel and run at 100V (LKB Bromma 2301, Pharmacia). When the loading buffer had travelled approximately 5cms through gel, the gel was removed, viewed under ultra-violet light and

photographed. PCR product with a strong band in the 1500bp region was considered adequate for use in a sequencing reaction (see section 2.9.1.5).

2.9.1.5. Sequencing of PCR Products
(using Applied Biosystems *Taq* DyeDeoxy Terminator Sequencing Kit)

The approximate DNA concentration of the PCR products was determined by comparison of fluorescence intensity of the PCR products with that of molecular weight markers of known concentration.

The following premix solution, suitable for 10 reactions, was made up in a 1.5 mL microfuge tube:

5X TACS buffer	40 µL
dNTPs	10 µL
A-Dye Terminator	10 µL
C-Dye Terminator	10 µL
G-Dye Terminator	10 µL
U-Dye Terminator	10 µL
Taq polymerase	2.5 µL
Total	92.5 µL

The following solutions were added in the listed order to a 0.6 mL microfuge tube:

Premix (as above)	9.25 µL
Primer*(25ng/µL)	1.0 µL
PCR product	x†
Water	9.75 - x
Total	20 µL

* The primer used was dependant on the sequence to be determined. The primers shown in Table 2.3 were used to sequence different segments of the 16S rDNA molecule

Table 2.3: Primers used for sequencing of 16S rDNA

Primer	Binding region*	Primer Sequence (5' to 3')
530 reverse	519-536	G(T/A)ATTACCGCGGC(T/G)GCTG
Fox forward	786-803	ATTAGATACCCTGGTAG
1100 forward	1100-1115	GCAACGAGCGCAACCC

* Number is based on the *E. coli* numbering system from Brosius *et. al.*, 1981

† Amount or PCR product added was dependant on concentration present following PCR and purification steps.

The tubes were centrifuged (Eppendorf benchtop centrifuge 5415) for 1 minute at 14000 rpm and the solution overlayed with 80 µL of sterile light white mineral oil (Sigma). The tubes were then placed in the thermal cycler (Perkin Elmer DNA Thermal Cycler 480), heated to 98°C for 2 minutes and 0.4 µL *Taq* polymerase (Boehringer, 5U/µL) was added below the oil overlay. The thermal cycler was then run through the following profile:

28 cycles:	96 °C	30 sec.
	50 °C	15 sec.
	60 °C	4 min.

cool to 4°C

The samples were then removed from the thermal cycler and stored at 4°C prior to purification.

2.9.1.6. Purification of Sequencing Reactions

A microfuge tube containing sequencing reaction product (section 2.9.1.5) stored at 4°C was allowed to come to room temperature. 80 µL sterile HPLC grade water was added below the oil overlay and the 100 µL removed to a fresh tube leaving behind the oil. An equal volume of phenol:chloroform:water 34:7:9 mixture was added to the tube, which was vortexed and then centrifuged (Eppendorf benchtop centrifuge 5415C) at 14000 rpm for 10 minutes. The top layer was removed to a new tube and the addition of phenol:chloroform:water, vortexing, centrifugation and removal to a new tube was repeated as above. 300 µL of absolute ethanol and 9.5 µL of 3M sodium acetate were added and the tube was placed on ice

for 30 minutes, the tube was then removed and centrifuged at 14000 rpm for 20 minutes, at 4°C. The supernatant was decanted immediately the centrifuge stopped. 500 µL of 70% ethanol was added to the tube and it was centrifuged at 14000rpm for 10 minutes, at 4°C. The supernatant was again decanted immediately the centrifuge stopped. The tube was then placed in a Savant DNA Speed Vac set at "medium" rotation speed for 45 minutes after which it was removed and stored at -20°C prior to loading on a sequencing gel (see section 2.9.1.7).

2.9.1.7. 16S rDNA Sequencing Reaction Product Analysis

Sequencing reaction product (see section 2.9.1.6), stored at -20°C, was resuspended in 3 µL of Formamide (Kodak international Biotechnologies): 50mM EDTA (Sigma) (5:1).

The glass plates used for the gel were cleaned with ALCONOX detergent such that no marks were visible on the surfaces. The surfaces of the plates which were to come into contact with the gel were wiped again with 90% isopropanol. Once clean, the glass plates were prepared for the gel by sandwiching rubber strips between them and holding the plates in place with bulldog clips.

The following were added to a clean glass beaker:

Distilled water	22.5 mL
10 X TBE (Appendix 1)	6 mL
Polyacrylamide solution*	9 mL

*Appendix 1

The beaker was then put on a hotplate and stirred at low speed on low heat (below 50°C), and 30 g of urea (Merck) was added. This was stirred until the urea had dissolved after which the solution was filtered through a 0.2 µm filter (Sartorius) into a clean beaker. 180 µL of ammonium persulphate and 24 µL of N, N, N', N'-tetramethylethyldiamide (TEMED, Kodak international Biotechnologies) was added and stirred thoroughly. The solution was now poured into the opening between the two plates so that it filled the space between the plates leaving no air bubbles. The plates were then placed on a horizontal surface, the open ends were closed with rubber seals, and the gel was left to set for two hours at room temperature. Once

the gel had set the rubber seals were removed and any loose fragments of gel were removed by scraping with the rubber seal and washing the outside of the plates with de-ionised water. The bulldog clips were removed and the plate was placed into the Applied Biosystems 373A DNA Sequencer with a 1 X TBE buffer (Appendix 1) in the upper and lower reservoirs. The plate was scanned to ensure that no marks on the glass would interfere with the reading of the sequence. Any marks found on the plates were removed with 90% isopropanol. The loading comb was placed in the top of the gel to a depth of approximately 1 mm. A pre-run was performed to ensure that all settings were as follows:

Voltage	1400-1600V
Amps	20mAMP
Watts	30W

The resuspended sequence reaction product was placed into a thermal cycler (Perkin Elmer DNA Thermal Cycler 480) and heated to 90°C for two minutes and then placed on ice. The samples were then loaded into the sample wells at the top of the gel and the current was again applied. The sequence was analysed using GeneScan-672 Software on an Apple Macintosh. The run length was 12 hours.

2.9.2 16S rDNA-23S rDNA Spacer Analysis

2.9.2.1 Culturing of Isolates and Extraction of DNA

Culturing of isolates, extraction of DNA and purification by prep-a-gene 16SrRNA, see methods 9.1.1-9.1.4 above.

2.9.2.2 Polymerase Chain Reaction (PCR)

Reagents were added to a sterile 0.6 mL microfuge tube in the following order:

10X incubation buffer (Boehringer)	10 µL
1mM dNTPs (Boehringer)	20 µL
primer: AM 1500 F (0.4g/L)	1 µL
primer: 23S R (0.3g/L)	1 µL
template DNA*	2 µL
Water	65.6 µL
Total volume	99.6 µL

* The template DNA prepared and purified as in sections 9.1.1 and 9.1.2. It was centrifuged for 1 minute at 14000 rpm immediately before use to ensure no Matrix was present in the reaction mixture.

The mixture was vortex mixed for 1 minute and centrifuged (Eppendorf benchtop centrifuge 5415C) at 14000 rpm for 1 minute, then overlayed with 80 µL of sterile light white mineral oil (Sigma) and placed in the thermal cycler (Perkin Elmer DNA Thermal Cycler 480). The tube was heated to 94°C for three minutes and 0.4 µL *Taq* polymerase (Boehringer, 5U/µL) was added below the oil overlay. The thermal cycler was then run through the following profile:

15 cycles:	48°C	30 sec.
	72°C	1 min.
	94°C	30 sec.
final cycle:	56°C	30 sec.
	72°C	5 min.

cool to 4°C

The final product was stored at 4°C prior to gel electrophoresis.

2.9.2.3 Gel Electrophoresis

Gel electrophoresis was performed as outlined in section 9.1.7 with the following modifications.

1/50 and 1/100 dilutions of PCR products were made in water.

A loading Premix was made with the following volumes per sample:

Formamide	1.2 µL
Size Standard (GeneScan 2500)	0.3 µL
Loading Dye (Appendix 1)	0.3 µL

Each of the two sample dilutions were mixed with the premix in the following volumes:

Premix	1.8 µL
Diluted PCR product	1.2 µL

The run length was 5 hours and 30 minutes, and samples were run with GeneScan-2500^{TM} ROX to provide standards for determination of fragment size.

2.9.3 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

2.9.3.1 Choice of Restriction Endonucleases

Sites of known 16S rDNA sequence difference between five Antarctic isolates were compared to the recognition sites for 27 commercially available restriction endonucleases (Table A.3.1, Appendix 3). Based on these criteria four enzymes were selected for use, *Hin* P1 I, *Aci* I, *Tsp* 45 I and *Tru* 9 I.

2.9.3.2 Restriction Digestion

Reagents and incubation conditions for each of four endonucleases used were as follows. Each of the reagents was added in the given order and then mixed well by taking up and ejecting from a pipette five or six times.

	Hin P1 I [1]	*Aci* I[3]	*Tsp* 45 I[5]	*Tru* 9 I[8]
Buffer	2.0 µL[2]	2.0 µL[4]	2.0 µL[6]	2.0 µL[9]
Water	12.75 µL	12.75µL	12.3µL	12.75 µL
Sample DNA	5.0 µL	5.0 µL	5.0 µL	5.0 µL
Enzyme	0.5 µL	0.5 µL	0.5 µL	0.25 µL
BSA	0 µL	0 µL	0.2 µL[7]	0 µL
Total vol.	20.25 µL	20.25 µL	20.0 µL	20.0 µL
Incubation conditions	37°C 2hrs	37°C 2hrs	65°C 2hrs	65°C 2hrs

1. New England Biolabs, 10 000 U/mL, recognition site: 5' G↓CGC 3'
2. New England Biolabs NEBuffer 2, (1 x concentration) see appendix 1 for composition.
3. New England Biolabs, 5000 U/mL, recognition site: 5' C↓CGC 3'
   3' GGC↑G 5'
4. New England Biolabs NEBuffer 3, (1 x concentration) see appendix 1 for composition.
5. New England Biolabs, 4000 U/mL, recognition site: 5' ↓GT(GC)AC 3'
6. New England Biolabs NEBuffer 1, (1 x concentration) see appendix 1 for composition.
7. Bovine Serum Albumin, New England Biolabs, 200µg/mL.
8. Boehringer Mannheim, 10 000 U/mL, recognition site: 5' T↓TAA 3'
   3' AAT↑T 5'
9. Boehringer Mannheim, SuRe/Cut Incubation Buffer M (1 x concentration) see appendix 1 for composition.

2.9.3.3 Analysis of Restriction Digestion by Gel Electrophoresis

The electrophoresis gel was prepared as in section 9.1.4 except for the following changes:

Agarose *	3%
Sample Volume	25 µL
Loading Buffer (Appendix 1)	5 µL
Molecular weight Marker†	5 µL

*Boehringer Mannheim, Agarose MS
†Boehringer Mannheim, VIII

The gel was placed in the electrophoresis apparatus (Gibco Br1 Horizon) containing X1 TBE buffer (Appendix 1) sufficient to cover the gel and placed in a 4°C refrigerator. Each sample pipetted into its own well in the gel and the voltage set at 100V (LKB Bromma 2301, Pharmacia). The current was turned off when the loading buffer had travelled approximately 8 cms through gel, a run time of about 5 hours. The Gel was then removed, viewed under ultra-violet light (UV Transilluminator, UVP Inc.) and photographed.

2.9.4 DNA-DNA Hybridization and Determination of GC%

DNA extraction and concentration was based on the procedure of Cashion *et al.*, (1977).

2.9.4.1 Culturing and Harvesting of Cells

Isolates were maintained on slopes of Sporulation medium (Appendix 1). A loopful of aerial mycelia was used to inoculate 250 mL of Glucose-Yeast-Malt (GYM) Broth (Appendix 1) in a 500 mL flask. The cultures were incubated at 25°C in a orbital incubator-shaker at 100 revolutions per minute. Cultures were checked for purity at maximum growth, harvested by centrifugation and washed three times in distilled water. At least 1g wet weight of cells was required in order to proceed with the DNA extraction and purification.

2.9.4.2 Extraction of DNA

30mL of Urea Buffer (Appendix 1) was added to the cells which were then passaged three times through a French press at a cell pressure of 16000 atm.

2.9.4.3 Washing and Concentration of DNA

Binding to Hydroxyapatite

1.5 g of Hydroxyapatite (Bio-Rad Hydroxyapatite, DNA Grade (Biogel) was weighed out into a 250 mL beaker and 0.8 g into a 150 mL beaker. Twenty mL of Phosphate Buffer 1 (Appendix 1) was poured into each beaker. The beakers were then left to stand until the hydroxyapatite had settled and the buffer was decanted off and discarded. This washing of the hydroxyapatite was repeated once again with 20 mL of Phosphate Buffer 1 and once with 20 mL of Urea Buffer. The disrupted cells from the French press were poured into the 250 mL beaker and swirled until the hydroxyapatite formed a homogeneous suspension. The suspension was left to stand at room temperature for one hour, after which the hydroxyapatite had settled, and the supernatant was now decanted and discarded. The remaining hydroxyapatite was washed twice more in the manner described above, with 20 mL of Urea Buffer.

Washing and Concentration

A 10mL syringe was used as a separation column. The syringe nozzle was plugged with sufficient glass wool to prevent the passage of the hydroxyapatite while allowing the passage of solutions. 20mL of Phosphate Buffer 1 was added to the hydroxyapatite in the 150 mL beaker, it was swirled until the hydroxyapatite formed a homogeneous suspension and was then poured into the column. The level of solution in the column was not allowed to fall below that of the settled hydroxyapatite from that point onwards. The column was filled and rinsed once with Phosphate Buffer 1 and twice with Urea Buffer and then the hydroxyapatite from the 250mL beaker, to which the DNA had been bound, was added by swirling in Urea Buffer and pouring onto the column. The column was constantly refilled with Urea Buffer until the hydroxyapatite from the 250mL beaker had settled, it was then attached to a peristaltic pump and washed with Urea Buffer at 15mLs/hr for 10 hours. After the wash the Urea Buffer was

replaced with Phosphate Buffer 2 (Appendix 1). The outflow from the column was monitored at 260nm as an indicator of the presence of DNA. Fractions of the outflow were collected in clean tubes. Fractions which showed a high absorbance at 260nm were pooled and transferred to dialysis tubing. The tubing was placed in a 0.1%SSC (Appendix 1) solution, which was slowly stirred at 4°C overnight. After dialysis the pooled fraction was transferred to a fresh tube and stored at -20°C.

3mL of the fraction following the dialysis step was passaged three times through a French press at a cell pressure of 30 000 atm. This solution was transferred to dialysis tubing and placed in a 2.5% SSC solution (Appendix 1), and was slowly stirred with a magnetic stirring bar, and left for two days at 4°C. The buffer was changed twice a day during dialysis and the final buffer was retained for use as a blank in the hybridisation step.

2.9.4.4 Hybridization

DNA-DNA hybridization was based on a method by DeLey *et al.*, (1970), modified for use with a GilfordSystem 2600 Spectrophotometer equipped with a Gilford 2527-R thermoprogrammer and plotter.

DNA concentration was adjusted to 45 µg/mL. Because Streptomyces have a high GC content 20% dimethyl sulphoxide (DMSO) (Escara and Hutton, 1980) was added to 2.5xSSC (Appendix 1) to lower the melting point (Tm) of the DNA. The resulting buffer was 2xSSC+20% DMSO. The average Tm was determined from the DNA melting curves and the optimal temperature of renaturation (TOR) was calculated from the formula TOR = Tm-25.

Renaturation rates were calculated by regression analysis between 10 and 30 minutes after the start of the reaction using the computer program TRANSFER.BAS (Jahnke, 1991). The homology values were calculated by the formula $H\% = 100(4xVM-VA-VB)/(2x\sqrt{[VAxVB]})$
where:
H% = % homology
VM = initial renaturation rate of mixture of samples A and B
VA = initial renaturation rate of sample A
VB = initial renaturation rate of sample B

2.9.4.5 Determination of GC%

GC% was determined by temperature of denaturation (Owen and Pitcher, 1985) which is related to GC%. Tm for 45µg/l DNA in SSC buffer was determined using a GilfordSystem 2600 Spectrophotometer equipped with a Gilford 2527-R thermoprogrammer and plotter, using the double derivation of the plot to calculate the Tm. The following formula was then used:

(G+C)% = 2.44Tm-169.00*

*From De Ley *et al.*, (1970).

Chapter 3: Results

3.1 Isolation

Twenty-four soils were collected from Antarctica. Inoculation streaks from six of the 24 soils developed microcolonies of hyphal actinomycetes (Table 3.2). Fifty-two isolates with actinomycete morphology were recovered using the Skerman micromanipulator. Soil sample 16 had a single hyphal colony but this was not recovered due to poor growth and overwhelming fungal contamination. Not all microcolonies could be recovered because: i) not all colonies isolated by micromanipulation grew when material was taken from the original colony and moved to a new site, ii) fungi or gliding bacteria or both eventually covered the surface of the plates making axenic isolation impossible. Microcolonies of hyphae-forming actinomycetes were easily distinguished from microcolonies of fungus as the latter had considerably thicker hyphae and formed less dense colonies.

Actinomycetes were most numerous on plates of 0.01% yeast extract when the soil inoculum had not undergone heat treatment (Table 3.1). The use of seawater based medium prevented actinomycete microcolony development from all soils except soil "9" from which six microcolonies developed.

Table 3.1 : Comparative abundance of actinomycete, other bacteria and fungal microcolonies grown on 0.01% yeast extract agar and seawater 0.01% yeast extract agar for 45 days at 10°C. One lot of inoculum was heat treated at 50°C for 10 minutes and plated onto 0.01% yeast extract agar. The inoculum was a 25 µL aliquot of 1 mL of water in which 0.1 g of the original soil had been vortex mixed, approximating a x400 dilution of the soil.

Abundance of microcolonies observed for each treatment

Soil No.	0.01% yeast extract agar			Seawater 0.01% yeast extract agar			Heat treatment 0.01% yeast extract agar		
	Actino-mycetes	Other Bacteria	Fungi	Actino-mycetes	Other Bacteria	Fungi	Actino-mycetes	Other Bacteria	Fungi
1	0	0	0	0	++	0	0	0	0
2	0	++	0	0	++	0	0	0	0
3	0	+	0	0	+	0	0	0	0
4	0	+++	0	0	+++	0	0	0	0
5	0	+	0	0	+++	+++	0	0	0
6	0	+	0	0	+	0	0	0	0
7	0	0	0	0	++	0	0	0	0
8	0	0	0	0	++	++	0	0	0
9	++	0	+	+	+	0	0	0	0
10	0	0	0	0	++	0	0	0	0
11	0	+	0	0	+++	++	0	0	0
12	0	+	++	0	+++	+	0	0	0
13	0	+++	0	0	+++	+++	0	0	0
14	0	++	0	0	+++	0	0	0	0
15	0	+++	0	0	+++	0	0	0	0
16	+	++	+	0	+	++	0	0	0
17	+	+++	++	0	++	++	0	0	0
18	++	++	++	0	++	++	0	0	0
19	++	+++	0	0	++	+	+	0	0
20	0	+	+	0	+	0	0	0	0
21	0	++	+	0	+	+	0	+	0
22	+	+	+	0	++	+	+	0	0
23	0	0	0	0	+	0	0	0	0
24	0	0	0	0	+++	0	0	0	0

+ = 1-10 colonies, ++ = 10-100 colonies, +++= greater than 100 present

Pre-treatment of soil:water suspensions with heat (50°C for 10 minutes) decreased numbers of actinomycete microcolonies to zero in all except soils 19 and 22. It also removed all other viable bacteria and fungi that were culturable on the isolation media, from all samples except soil 21. One actinomycete microcolony developed from the heat treated soil 22 inoculum and was assigned strain number "22c". Two actinomycete microcolonies developed from the heat treated soil 19 inoculum and were assigned numbers "19j" and "19k".

3.2 Characteristics of Soils From which Actinomycetes were Isolated

The pH, electro-conductivity (as an indication of salinity), percentage water by weight (%Water) and percentage loss of weight of dried soil on ignition at 600°C (%LOI) were determined for each soil sample (Table 3.2). These results were compared with numbers of actinomycete microcolonies that developed from each soil sample. The presence of macroscopic life at the sites from which soil samples were taken was also noted (Table 3.2).

There was good correlation between the presence of moss or lichen or both at the sample site and the development of actinomycetes on the inoculation streak. For every sample where moss or lichen was observed at the collection site (soils 9, 16, 17, 18, 22) actinomycete microcolonies developed in the inoculation streak. The only actinomycete bearing soil that was not associated with moss or lichen was one at which human disturbance had occurred (soil 19).

Actinomycetes were not isolated from sites that contained photosynthetic oragnisms other than moss and lichen. These included sites four and five where there were green algae, and site 23 where there was an algal mat. Actinomycetes were not isolated from any soils associated with penguins (soils 2, 7, 8, 11, 12, 13, 14, 15 and 23).

Table 3.2: Some characteristics of Antarctic soil samples. pH, Electro-conductivity (EC), %loss of weight of dried soils at 600°C, % water (w/w) and presence of macroscopic life for 24 Antarctic soil samples compared with numbers of actinomycete microcolonies that developed in the inoculation streak. The inoculum was a 25µL aliquot of 1mL of water in which 0.1g of the original soil had been vortex mixed, approximating a x400 dilution of the soil.

Sample number	No. of colonies	pH	EC (mS cm^{-1})	% LOI	%Water	Macroscopic life
1	0	7.2	0.31	0.11	1.4	None observed
2	0	7.5	3.06	1.08	6.0	Two penguins nearby. Feather next to sample area.
3	0	6.7	10.16	1.47	1.8	None observed
4	0	8.0	0.86	3.73	12.1	Beneath rock with green algae
5	0	8.6	1.21	1.89	6.5	Beneath rock with green algae
6	0	7.7	2.58	3.17	12.9	Occasional humans and flying birds
7	0	6.8	1.77	0.56	8.8	Penguins common
8	0	7.5	19.35	3.83	12.8	Penguins common
9	**39**	**7.1**	**0.13**	**0.49**	**1.1**	Black lettuce lichen nearby
10	0	7.3	0.31	0.68	5.9	None observed
11	0	5.7	28.23	59.23	50.2	Penguins very common
12	0	6.2	14.52	70.33	8.6	Penguins nearby
13	0	6.6	0.27	4.01	23.4	Penguins nearby
14	0	6.5	1.61	26.44	57.1	Penguins very common
15	0	7.0	0.05	0.32	0.2	Penguins common
16	**1**	**5.9**	**0.08**	**0.96**	**1.2**	Dried out moss and lichen bed, observed green in Jan. 1988
17	**6**	**7.8**	**0.42**	**16.98**	**37.0**	Moss and lichen bed
18	**73**	**7.3**	**0.28**	**5.85**	**13.5**	Moss and lichen bed
19	**24**	**6.4**	**0.10**	**0.55**	**5.1**	Vehicle and foot tracks.
20	0	7.0	1.63	6.36	43.1	None observed
21	0	5.6	0.16	6.68	2.3	None observed
22	**3**	**7.4**	**0.68**	**2.18**	**8.6**	Well developed moss and lichen beds
23	0	7.7	2.13	1.42	0.8	Algal mat, a few penguins
24	0	7.8	26.61	16.11	33.6	None observed

There does not appear to be any correlation between soil pH and the presence of actinomycetes (Tables 3.2, 3.3), although the range of soil pH was limited.

Electro-conductivity (EC) of soils from which actinomycetes were isolated was consistently lower (ranging from 0.08 to 0.68 mS cm^{-1}) than the average (4.85 mS cm^{-1}) (Tables 3.2, 3.3), even when ornithogenic soils, which had a higher than average EC, were excluded from the calculation of the average (2.83 mS cm^{-1}).

%Loss of weight (%LOI) of a dried soil sample following a 600°C heat treatment indicates the proportion of organic carbon present in the soil. %LOI was on average lower for soils from which actinomycetes were cultivated, but when ornithogenic soils were excluded from the calculation of the average, the difference in %LOI between soils from which actinomycetes were recovered and other soils was negligible (Table 3.3). Actinomycetes occurred in soils with a %LOI which ranged from relatively high, at 16.98% (soil 17) to relatively low, at 0.49% (soil 9).

Table 3.3: Comparison of average values for pH, Electro-conductivity (EC), %loss of weight of dried soil at 600°C (%LOI) and %water (w/w) for different categories of Antarctic soils.

Soils for which average is calculated	pH	EC (mS)	% LOI	% water
All soils	7.04	4.85	9.77	14.75
All soils from which actinomycetes were isolated	6.96	0.28	4.50	11.08
Ornithogenic soils	6.86	8.90	20.62	19.58
All except ornithogenic soils	7.13	2.83	4.34	12.34
All except ornithogenic soils and those from which actinomycetes were isolated	7.24	4.37	4.25	13.09

Soils in which actinomycetes occurred had a similar %water to the average (Table 3.3). The variation in water contents in the actinomycete-bearing soils was from 1.1% (soil 9) to 37.0% (soil 17). Therefore %water in soil

within this range was not a critical factor for actinomycete survival at the time of sampling but may be critical at other times.

3.3 Morphology and Physiology

Colony morphology was not a stable characteristic for each isolate. Many colonies produced a pigment only once over three or four sub-cultures on an identical medium. For example, all isolates from soil 19 produced a very strong black-purple pigment on sporulation media from at least one sub-culture but most of these isolates produced a weak brown-grey pigment from a different sub-culture on the same medium. Other physiological and morphological attributes which were not consistently expressed were aerial hyphae production, spore production, spore chain morphology, antibiotic production and amount of melanin produced.

Results shown in Tables 3.4 and 3.5 were obtained through multiple observations except antibiotic production which was only tested once for each isolate.

Table 3.4: Appearance of actinomycete colonies after incubation at 25°C for three weeks on Sporulation agar, GYM agar and Starch agar.

	Colony Description								
	Sporulation Agar			GYM Agar			Starch Agar		
Isolate No.	Upper Surface	Lower Surface	Diff-usible Pigment	Upper Surface	Lower Surface	Diff-usible Pigment	Upper Surface	Lower Surface	Diff-usible Pigment
9a	white	orange	brown	white/grey	grey/yellow	dark brown	thick, white	yellow/brown	brown
9c	white	clear	none	ND	ND	ND	ND	ND	ND
9d	white	clear	brown	no spores	brown	brown	no spores	cream	none
9e	white	clear	none	white	brown	brown	yellow	cream/brown	none
9f	violet	yellow	none	ND	ND	ND	ND	ND	ND
9g	violet	yellow*	none	ND	ND	ND	ND	ND	ND
9h	white	yellow	none	ND	ND	ND	ND	ND	ND
9i	violet	yellow*	none	ND	ND	ND	ND	ND	ND
9j	violet	yellow	none	ND	ND	ND	ND	ND	ND
9k	violet	yellow	none	ND	ND	ND	ND	ND	ND
9l	violet	yellow	none	ND	ND	ND	ND	ND	ND
9n	violet	yellow	brown	ND	ND	ND	ND	ND	ND
9o	violet	yellow	none	ND	ND	ND	ND	ND	ND
9q	violet	yellow	none	violet	yellow	none	white/pink	cream	none
9r	brown	yellow/clear	none	ND	ND	ND	ND	ND	ND
9s	white	yellow	brown	ND	ND	ND	ND	ND	ND
9t	brown	orange/brown	olive	cream/grey	tan	grey/brown	yellow/olive	dark olive	light brown
9v	violet	yellow*	none	ND	ND	ND	ND	ND	ND
17a	violet	yellow	none	ND	ND	ND	ND	ND	ND
17b	white	yellow*	none	ND	ND	ND	ND	ND	ND
17c	violet	yellow	brown	ND	ND	ND	ND	ND	ND
18a	violet	yellow*	brown	violet	yellow	yellow	violet	cream	none
18b	violet	yellow	none	ND	ND	ND	ND	ND	ND
18c	violet	yellow*	brown	ND	ND	ND	ND	ND	ND
18e	violet	yellow	none	ND	ND	ND	ND	ND	ND
18f	violet	yellow*	none	ND	ND	ND	ND	ND	ND
18h	violet	yellow*	none	ND	ND	ND	ND	ND	ND
18j	white	yellow*	brown	ND	ND	ND	ND	ND	ND
18l	violet	yellow	brown	ND	ND	ND	ND	ND	ND
18n	violet	yellow*	brown	ND	ND	ND	ND	ND	ND

	Sporulation Agar			GYM Agar			Starch Agar		
Isolate No.	Upper Surface	Lower Surface	Diff-usible Pigment	Upper Surface	Lower Surface	Diff-usible pigment	Upper Surface	Lower Surface	Diff-usible Pigment
18o	violet	yellow*	none	ND	ND	ND	ND	ND	ND
18p	violet	yellow	none	ND	ND	ND	ND	ND	ND
18r	violet	yellow*	brown	ND	ND	ND	ND	ND	ND
18s	violet	yellow*	none	ND	ND	ND	ND	ND	ND
18t	violet	yellow	brown	white	yellow	yellow	white	cream	none
18x	white	yellow	brown	ND	ND	ND	ND	ND	ND
19a	grey	olive	grey	ND	ND	ND	ND	ND	ND
19b	grey	olive	brown	grey	purple/ brown	brown	grey	yellow/ brown	none
19c	grey	olive	none	ND	ND	ND	ND	ND	ND
19e	grey	olive	none	grey	brown	light brown	grey	brown/ purple	none
19g	grey	olive	none	grey	purple/ brown	brown	grey	yellow/ brown	ND
19j	grey	olive	brown	ND	ND	ND	ND	ND	ND
19k	grey	olive	brown	grey	purple/ brown	brown	grey	yellow/ brown	none
19l	grey	olive	none	ND	ND	ND	ND	ND	ND
19n	grey	olive	none	ND	ND	ND	ND	ND	ND
22a	violet	yellow	none	ND	ND	ND	ND	ND	ND
22b	white	yellow	none	ND	ND	ND	ND	ND	ND
22c	white	orange	brown	ND	ND	ND	ND	ND	ND
S9a	white	orange	brown	ND	ND	ND	ND	ND	ND
S9b 2	brown	yellow	brown	white	brown	brown	cream	cream	none
S9c	white	orange	brown	ND	ND	ND	ND	ND	ND
S9d	white	clear	none	no spores	brown	brown	no spores	yellow	none

* = fluorescent under UV light (indicates presence of gilvocarcin)
ND = Test not done

Table 3.5: Spore chain morphology, and production of antibiotics and melanin on peptone-yeast extract-iron agar (PYI) by the Antarctic isolates. Antibiotic production was indicated by a zone of inhibition around actinomycete colonies grown on sporulation agar for four days at 25°C and then overlaid with a lawn of one of four test organisms, *Staphylococcus aureus Pseudomonas aeruginosa, Bacillus subtilis* or *Escherichea coli.*

Isolate number	Radius of inhibition in mm from the edge of the actinomycete colony of test organisms				Spore chain morphology	Melanin on PYI agar
	Staph. aureus	*Ps. aeruginosa*	*B. subtilis*	*E. coli*		
9a	0	0	0	0	Reflexibles	no
9c	0	0	0	0	Reflexibles	no
9d	0	0	0	0	No sporing bodies seen	yes
9e	0	0	0	0	No sporing bodies seen	yes
9f	4	9	10	8	Retinaculiaperti	no
9g	8	5	12	10	Retinaculiaperti	no
9h	6	0	0	0	Retinaculiaperti	no
9i	4	0	0	0	Retinaculiaperti	no
9j	10	5	5	0	Retinaculiaperti	no
9k	10	5	10	5	Reflexibles	no
9l	12	2	10	4	Retinaculiaperti	no
9n	8	0	6	0	Retinaculiaperti	no
9o	9	0	0	0	Reflexibles	no
9q	0	3	9	7	Retinaculiaperti	no
9r	0	0	0	0	Retinaculiaperti	no
9s	0	2	9	8	Retinaculiaperti	no
9t	7	0	0	0	Retinaculiaperti	no
9v	2	2	6	6	Retinaculiaperti	no
17a	0	0	0	7	Retinaculiaperti	no
17b	10	5	4	8	Reflexibles	no
17c	20	6	15	0	Reflexibles	no
18a	8	5	9	10	Retinaculiaperti	no
18b	10	0	0	0	Reflexibles	no
18c	4	5	12	9	Retinaculiaperti	no
18e	1	0	5	5	Retinaculiaperti	no
18f	15	3	20	7	Retinaculiaperti	no
18h	0	5	14	9	Retinaculiaperti	no
18j	7	0	7	9	Retinaculiaperti	no
18l	10	0	6	10	Retinaculiaperti	no
18n	12	3	15	10	Retinaculiaperti	no
18o	5	5	15	10	Retinaculiaperti	no

Isolate number	Radius of inhibition in mm from the edge of the actinomycete colony of test organisms				Spore chain morphology	Melanin on PYI agar
	Staph. aureus	*Ps. aeruginosa*	*B. subtilis*	*E. coli*		
18p	9	0	8	0	Retinaculiaperti	no
18r	10	0	4	8	Retinaculiaperti	no
18s	10	9	15	10	Reflexibles	no
18t	10	4	10	2	Retinaculiaperti	no
18x	11	5	9	2	Retinaculiaperti	no
19a	0	0	3	0	Spirales	yes
19b	0	0	0	0	Spirales	yes
19c	0	0	0	0	Spirales	yes
19e	0	0	0	0	Spirales	yes
19g	0	0	0	0	Spirales	yes
19j	0	0	0	0	Spirales	yes
19k	0	0	0	0	Spirales	yes
19l	0	0	0	0	Spirales	yes
19n	0	0	0	0	Spirales	yes
22a	7	0	5	5	Reflexibles	no
22b	0	0	11	7	Retinaculiaperti	yes
22c	0	0	0	0	Reflexibles	no
IS9a	0	0	0	0	Retinaculiaperti	no
IS9b2	0	7	0	0	Retinaculiaperti	no
IS9c	0	0	0	0	Retinaculiaperti	no
IS9d	0	0	0	0	No sporing bodies seen	yes

3.4 Production of Antibiotics

An antibiotic produced by isolate 18a was identified as gilvocarcin (Dragar, C., unpublished data). This antibiotic was yellow, fluoresced under ultra-violet light, and was an unstable diffusible pigment.

18a was phenotypically compared to other actinomycete strains known to produce gilvocarcin but it differed from each of the five reported strains (Table 3.7).

An antibiotic produced by isolate 9t was tentatively identified as a member of the ansamycin family of antibiotic compounds (Carver, 1993). The antibiotic produced by 9t differed from all described structures in the ansamycin family of antibiotic compounds (Carver, 1993), and has probably not been described previously, but insufficient data were collected to fully describe the structure of this antibiotic. The antibiotic produced by strain 9t was reported to be most similar to streptovaricins and rifamycins, sub-classes of the ansamycin family (Carver, 1993).

3.5 Numerical Taxonomy

Data on each strain's spore mass colour, spore chain morphology, melanin production on peptone-yeast extract-iron (PYI) agar, antibiotic production against four test organisms and ability to grow on sole carbohydrates was coded in binary format (Table 3.8). Characters which had more than two states such as spore chain morphology and spore mass colour were coded as multi-state variables, which allowed these characters to be used without giving them unequal weighting compared to binary characters. All isolates were compared by Burr's Strategy (Ross and Shields, 1993) using the software TAXON (Ross and Shields, 1993). A tree showing relatedness between isolates based on the results of this comparison was constructed (Figure 3.1). Each of the isolates fell into one of four well defined groups. These four groups were designated A, B, C and D. Strains typical of these groups were 9a and 9e (group A), 9t (group B), 18a (group C) and 19k (group D).

Table 3.6: Growth of Antarctic isolates on sole carbon sources. Isolates with the same patterns of growth are placed in the same column. Extent of growth is indicated as follows: "-" = no growth, 1 = low growth, 2= medium growth, 3=high growth.

Sole carbon source	Isolates																														
	9a	9c	9r	22c	S9c	S9a	9e	9d S9d	9t	S9 b2	*S. gr.*	9f 9g 9i 9l	9h 9o 18f 18l 18x	9j 9k 9n 9s 18b 18c 18h 18n 22b	9q 17a 18a 18o	9v	17c 18j	17b	18e 18r 18s 18t	18p	22a	19a	19b	19c	19e	19g	19j	19k	19l	19n
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	2	3	3	3	3	3	3	3	3	3	-	3	3	3	3	3	3	3	3	3	3	3	3	3	2	2	3	3	3	3
Cellobiose	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Dextran	-	-	-	-	-	-	-	-	-	3	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fructose	2	3	2	2	2	2	3	3	1	2	1	-	1	-	1	1	-	-	-	1	1	1	-	-	1	1	-	-	1	-
Galactose	2	1	1	2	3	3	2	3	1	3	1	1	-	-	-	1	1	-	-	1	1	3	3	1	2	3	3	2	3	3
Glucose	3	3	3	2	3	3	3	3	3	3	3	3	3	3	3	3	2	3	3	3	3	3	2	3	3	3	3	3	3	3	3
Inulin	1	1	1	-	1	-	1	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1	-	-	1	-	-	1
Lactose	-	3	3	2	-	-	-	3	3	1	-	-	-	-	-	-	-	-	-	-	-	-	2	2	3	1	3	3	3	2	-
Mannitol	-	-	-	-	-	-	-	-	3	3	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannose	2	3	3	2	3	3	3	3	2	3	3	3	3	3	3	3	2	3	3	3	3	3	3	2	-	2	-	-	2	3	3
Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	3	1	3	-	2	2	3	3
meso-Inositol	-	-	-	2	1	1	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	3	1	1	3	-	3	2	3
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	1	2	2	1	3	2	1
Rhamnose	2	3	3	3	3	2	1	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	3	3	2	2	1	2	2
Salicin	1	1	1	2	2	1	2	2	2	1	2	2	2	2	2	2	2	1	2	2	1	1	1	1	-	1	1	1	1	1
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	1	3	3	3	3	3	1	1	2	3	1	-	-	-	-	-	-	-	-	-	-	-	3	3	3	2	3	3	3	3	3
Xylose	2	2	3	2	2	2	2	3	2	2	1	2	-	2	2	-	-	-	-	2	2	3	3	3	3	-	3	3	3	3

Table 3.7: Comparison of some phenotypic characteristics of Antarctic isolate 18a, a producer of the antibiotic gilvocarcin, with those of other known gilvocarcin-producing actinomycetes. Characteristics which distinguish described strains from 18a are shown in bold print.

	18a[1]	*S.gilvotanereus*[1]	*S. anandii*[2]	*S. collinus*[2]	*S. arenae*[2]	S. griseoflavus[2]
Spore chain morphology	Retinaculiaperti	spirals	spirals(occR.flex or R.a.perti)	Retinaculiaperti (looped)	**spirals**	retimaculiaperti
Colour: spore mass	violet	camel/mustard	**white or yellow**	**grey**	**grey**	**83green 17red**
: hyphael mass	white/yellow	camel/mustard	yellow brown	yellow/brown, red/orange or violet	yellow/brown, red/orange or violet	yellow/brown or green blue
Pigment	yellow	none/rose/gold	**none**	yellow/brown, red/orange or violet	yellow/brown, red/orange or violet	none
Melanin on tyrosine	no	no	no	**yes**	**yes**	17
Utilization of						
Adonitol	-	ND	**100**	0	0	0
Arabinose	+++	-	71	89	89	100
Cellobiose	+++	ND	100	100	100	100
Dextran	-	ND	14	6	6	50
Fructose	+	+	86	56	56	83
Galactose	+++	ND	100	94	94	100
Inulin	+	ND	**0**	33	33	17
Lactose	-	ND	86	**94**	**94**	83
Mannitol	+	++	100	0	0	100
Mannose	++	ND	100	100	100	100
Melezitose	-	ND	57	22	22	**67**
Melibiose	-	ND	**100**	28	28	**67**
meso-Inositol	-	+	**100**	6	6	**83**
Raffinose	-	-	**86**	33	33	33
Rhamnose	-	++	0*	61	61	83
Salicin	+++	ND	86	100	100	67
Sucrose	-	+	0*	28	28	**83**
Trehalose	++	ND	100	50	50	100
Xylose	++	-	86	89	89	100

1 Sole carbon source utilization is presented as "-"= no growth, "+"=poor growth, "+"= medium growth, "+++"= good growth. 2 Sole carbon source utilization is presented as a number representing the % of strains in the cluster containing this strain which were positive for the given character (From Williams *et al.*, 1983a) References for production of antibiotics by the strains given above are: *S. "gilvotanareus"* , Nakano *et al.*, (1981); *S. anandii*, Balitz *et al.*, (1981); *S. collinus*, Horii *et al.*, (1980); *S. arenae*, Wei *et al.*, (1981); *S. griseoflavus* , Breiding-Mack and Zeeck (1986).

Table 3.8: Characters used in numerical taxonomic comparison. The characters were compared using TAXON, each character was equally weighted.

Strain number	9a	9c	9d	9e	9f	9g	9h	9i	9j	9k	9l	9n	9o
Colour Of Spore Mass[1]	2	2	2	2	1	1	2	1	1	1	1	1	1
Spore Chain Morphology[2]	1	2	0	0	2	2	2	2	2	1	2	2	1
Melanin Production on PYI agar	0	0	1	1	0	0	0	0	0	0	0	0	0
Antimicrobial Activity Against													
Staph. aureus	0	0	0	0	1	1	1	1	1	1	1	1	1
Ps. aeruginosa	0	0	0	0	1	1	0	0	1	1	1	0	0
B. subtilis	0	0	0	0	1	1	0	0	1	1	1	1	0
E. coli	0	0	0	0	1	1	0	0	0	1	1	0	0
Growth on sole carbon sources at 1% (w/v)													
Adonitol	0	0	0	0	0	0	0	0	0	0	0	0	0
Arabinose	1	1	1	1	1	1	1	1	1	1	1	1	1
Cellobiose	1	1	1	1	1	1	1	1	1	1	1	1	1
Dextran	0	0	0	0	0	0	0	0	0	0	0	0	0
Fructose	1	1	1	1	0	0	1	0	0	0	0	0	1
Galactose	1	1	1	1	1	1	0	1	0	0	1	0	0
Glucose	1	1	1	1	1	1	1	1	1	1	1	1	1
Inulin	1	1	1	1	0	0	0	0	0	0	0	0	0
Lactose	0	1	1	0	0	0	0	0	0	0	0	0	0
Mannitol	0	0	0	0	0	0	0	0	0	0	0	0	0
Mannose	1	1	1	1	1	1	1	1	1	1	1	1	1
Melezitose	0	0	0	0	0	0	0	0	0	0	0	0	0
Melibiose	0	0	0	0	0	0	0	0	0	0	0	0	0
meso-Inositol	0	0	1	0	0	0	0	0	0	0	0	0	0
Raffinose	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhamnose	1	1	1	1	0	0	0	0	0	0	0	0	0
Salicin	1	1	1	1	1	1	1	1	1	1	1	1	1
Sucrose	0	0	0	0	0	0	0	0	0	0	0	0	0
Trehalose	1	1	1	1	0	0	0	0	0	0	0	0	0
Xylose	1	1	1	1	1	1	0	1	1	1	1	1	0

Strain number	9q	9r	9s	9t	9v	17 a	17 b	17 c	18 a	18 b	18 c	18 e	18 f
Colour Of Spore Mass[1]	1	4	2	4	1	1	2	1	1	1	1	1	1
Spore Chain Morphology[2]	2	2	2	2	2	2	1	1	2	1	2	2	2
Melanin Production on PYI agar	0	0	0	0	0	0	0	0	0	0	0	0	0
Antimicrobial Activity Against													
Staph. aureus	0	0	0	1	1	0	1	1	1	1	1	1	1
Ps. aeruginosa	1	0	1	0	1	0	1	1	1	0	1	0	1
B. subtilis	1	0	1	0	1	0	1	1	1	0	1	1	1
E. coli	1	0	1	0	1	1	1	0	1	0	1	1	1
Growth on Sole Carbon sources at 1% (w/v)													
Adonitol	0	0	0	0	0	0	0	0	0	0	0	0	0
Arabinose	1	1	1	1	1	1	1	1	1	1	1	1	1
Cellobiose	1	1	1	1	1	1	1	1	1	1	1	1	1
Dextran	0	0	0	0	0	0	0	0	0	0	0	0	0
Fructose	1	1	0	1	1	1	0	0	1	0	0	0	1
Galactose	0	1	0	1	1	0	0	1	0	0	0	0	0
Glucose	1	1	1	1	1	1	1	1	1	1	1	1	1
Inulin	0	1	0	0	0	0	0	0	0	0	0	0	0
Lactose	0	1	0	1	0	0	0	0	0	0	0	0	0
Mannitol	0	0	0	1	0	0	0	0	0	0	0	0	0
Mannose	1	1	1	1	1	1	1	1	1	1	1	1	1
Melezitose	0	0	0	0	0	0	0	0	0	0	0	0	0
Melibiose	0	0	0	0	0	0	0	0	0	0	0	0	0
meso-Inositol	0	0	0	0	0	0	0	0	0	0	0	0	0
Raffinose	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhamnose	0	1	0	0	0	0	0	0	0	0	0	0	0
Salicin	1	1	1	1	1	1	1	1	1	1	1	1	1
Sucrose	0	0	0	0	0	0	0	0	0	0	0	0	0
Trehalose	0	1	0	1	0	0	0	0	0	0	0	0	0
Xylose	1	1	1	1	0	1	0	0	1	1	1	0	0

Strain number	18h	18j	18l	18n	18o	18p	18r	18s	18t	18x	19a	19b	19c
Colour Of Spore Mass[1]	1	1	1	1	1	1	1	1	1	2	3	3	3
Spore Chain Morphology[2]	2	2	2	2	2	2	2	1	2	2	3	3	3
Melanin Production on PYI agar	0	0	0	0	0	0	0	0	0	0	1	1	1
Antimicrobial Activity Against													
Staph. aureus	0	1	1	1	1	1	1	1	1	1	0	0	0
Ps. aeruginosa	1	1	0	1	1	0	0	1	1	1	0	0	0
B. subtilis	1	1	1	1	1	1	1	1	1	1	1	0	0
E. coli	1	1	1	1	1	0	1	1	1	1	0	0	0
Growth on Sole Carbon sources at 1% (w/v)													
Adonitol	0	0	0	0	0	0	0	0	0	0	0	0	0
Arabinose	1	1	1	1	1	1	1	1	1	1	1	1	1
Cellobiose	1	1	1	1	1	1	1	1	1	1	1	1	1
Dextran	0	0	0	0	0	0	0	0	0	0	0	0	0
Fructose	0	0	1	0	1	1	0	0	0	1	1	0	0
Galactose	0	1	0	0	0	1	0	0	0	0	1	1	1
Glucose	1	1	1	1	1	1	1	1	1	1	1	1	1
Inulin	0	0	0	0	0	0	0	0	0	0	1	1	1
Lactose	0	0	0	0	0	0	0	0	0	0	1	1	1
Mannitol	0	0	0	0	0	0	0	0	0	0	0	0	0
Mannose	1	1	1	1	1	1	1	1	1	1	1	1	0
Melezitose	0	0	0	0	0	0	0	0	0	0	0	0	0
Melibiose	0	0	0	0	0	0	0	0	0	0	1	1	1
meso-Inositol	0	0	0	0	0	0	0	0	0	0	1	1	1
Raffinose	0	0	0	0	0	0	0	0	0	0	0	1	1
Rhamnose	0	0	0	0	0	0	0	0	0	0	0	1	1
Salicin	1	1	1	1	1	1	1	1	1	1	1	1	1
Sucrose	0	0	0	0	0	0	0	0	0	0	0	0	0
Trehalose	0	0	0	0	0	0	0	0	0	0	1	1	1
Xylose	1	0	0	1	1	1	0	0	0	0	1	1	1

Strain number	19e	19g	19j	19k	19l	19n	22a	22b	22c	s9a	s9b2	s9c	s9d
Colour Of Spore Mass[1]	3	3	3	3	3	3	1	2	2	2	4	2	2
Spore Chain Morphology[2]	3	3	3	3	3	3	1	2	1	2	2	2	0
Melanin Production on PYI agar	1	1	1	1	1	1	0	1	0	0	0	0	1
Antimicrobial Activity Against													
Staph. aureus	0	0	0	0	0	0	1	0	0	0	0	0	0
Ps. aeruginosa	0	0	0	0	0	0	0	0	0	0	1	0	0
B. subtilis	0	0	0	0	0	0	1	1	0	0	0	0	0
E. coli	0	0	0	0	0	0	1	1	0	0	0	0	0
Growth on Sole Carbon sources at 1% (w/v)													
Adonitol	0	0	0	0	0	0	0	0	0	0	0	0	0
Arabinose	1	1	1	1	1	1	1	1	1	1	1	1	1
Cellobiose	1	1	1	1	1	1	1	1	1	1	1	1	1
Dextran	0	0	0	0	0	0	0	0	0	0	1	0	0
Fructose	1	1	0	0	1	0	1	0	1	1	1	1	1
Galactose	1	1	1	1	1	1	1	0	1	1	1	1	1
Glucose	1	1	1	1	1	1	1	1	1	1	1	1	1
Inulin	0	0	1	0	0	1	0	0	0	0	1	1	1
Lactose	1	1	1	1	1	0	0	0	1	0	1	0	1
Mannitol	0	0	0	0	0	0	0	0	0	0	1	0	0
Mannose	1	0	0	1	1	1	1	1	1	1	1	1	1
Melezitose	0	0	0	0	0	0	0	0	0	0	0	0	0
Melibiose	1	0	1	1	1	1	0	0	0	0	0	0	0
meso-Inositol	1	1	0	1	1	1	0	0	1	1	0	1	1
Raffinose	1	1	1	1	1	1	0	0	0	0	0	0	0
Rhamnose	1	1	1	1	1	1	0	0	1	1	0	1	1
Salicin	0	1	1	1	1	1	1	1	1	1	1	1	1
Sucrose	0	0	0	0	0	0	0	0	0	0	0	0	0
Trehalose	1	1	1	1	1	1	0	0	1	1	1	1	1
Xylose	1	0	1	1	1	1	1	1	1	1	1	1	1

[1] Spore colony coulours:*violet=1, white=2, grey=3, brown=4*

[2]Spore chain morphology: *Reflexibles=1, Retinaculiaperti=2, Spirales=3*

Table 3.9: Cramer values were derived for each character used in numerical taxonomy as an indication of reliability in separating clusters. A value of one indicates that this characteristic is consistent within all clusters. This value lessens as the character becomes less consistent within clusters. Characters are listed in descending order of Cramer values. These values were determined using TAXON software.

Character	Cramer value	Number of members in each cluster positive for this character. Total number of members is indicated in parenthesis.			
		C (32)	A (9)	D (9)	B (2)
Growth on Mannitol	1	0	0	0	2
Growth on Trehalose	1	0	9	9	2
Growth on Rhamnose	0.9604	0	9	8	0
Growth on Melibiose	0.9320	0	0	8	0
Growth on Raffinose	0.9320	0	0	8	0
Colour of Spore mass*	0.8475	27	8	9	2
Growth on Lactose	0.8417	0	5	8	2
Melanin Production on PYI agar	0.8340	1	3	9	0
Growth on *meso*-Inositol	0.8250	0	5	8	0
Antibiotic effect vs *Staph. aureus*	0.7968	27	0	0	1
Antibiotic effect vs *B. subtilis*	0.7777	27	0	1	0
Growth on Inulin	0.7492	0	7	5	1
Antibiotic effect vs *E. coli*	0.7319	24	0	0	0
Spore chain morphology†	0.7158	25	4	9	2
Growth on Galactose	0.7041	9	9	9	2
Growth on Dextran	0.7001	0	0	0	1
Antibiotic effect vs *Ps. aeruginosa*	0.6008	20	0	0	1
Growth on Mannose	0.5408	32	9	6	2
Growth on Fructose	0.5010	12	9	4	2
Growth on Xylose	0.3983	19	9	8	2
Growth on Salicin	0.3061	32	9	8	2

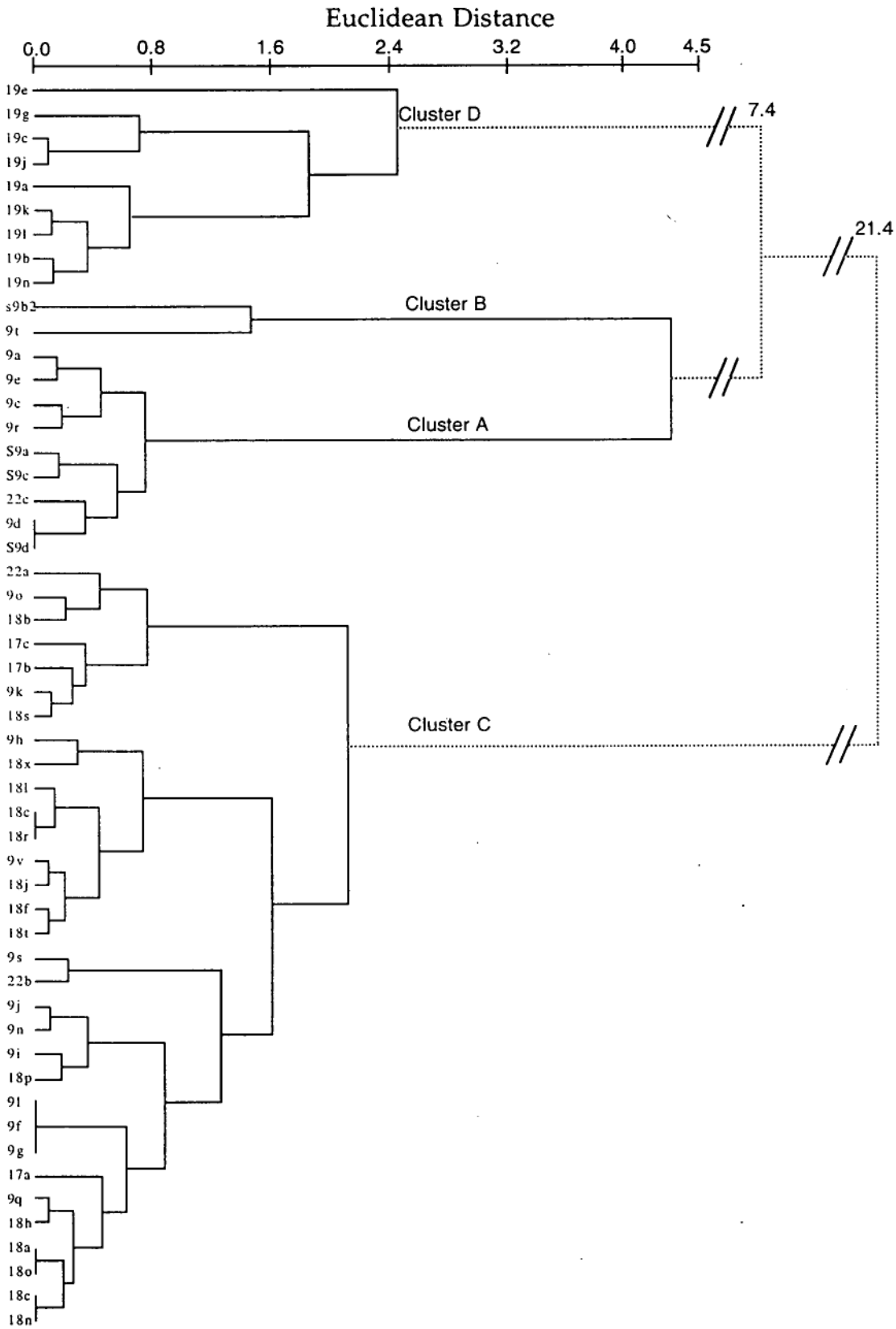
Growth on Adonitol, Melezitose and sucrose was negative for all isolates and growth on Arabinose, Cellobiose and Glucose was positive for all isolates so these characters are not included in this table as they have no value in differentiating between clusters.

* This was a multi-state variable, the spores could be one of four colours. The numbers given are the largest number of cluster members having the same colour of spore mass.

† This was a multi-state variable, the spore chains could show one of three morphologies. The numbers given are the largest number of cluster members showing the same chain morphology

The TAXON program also indicated *a postori* which of the characteristics were of most use in separating the isolates into the clusters it had determined (Table 3.9). Growth on mannitol was a characteristic found only in cluster C, and all strains of cluster C had this attribute. Inability to utilize trehalose was a characteristic exclusive to and consistent within cluster D. Utilization of mannitol and trehalose were the only two characters which were 100% consistent across and within clusters. Growth on rhamnose was consistently negative in clusters B and C and positive in clusters A and D with the exception of one isolate in cluster D (isolate 19a). Growth on melibiose and raffinose was consistently negative in clusters A, B and C and positive in cluster D with the exception isolate 19a which did not grow on raffinose and isolate 19g which did not grow on melibiose. Other characteristics were much less consistent within and between clusters. Some characters were the same for all strains (see notes to Table 3.9) and could not be used to differentiate between isolates.

Figure 3.1: Phenotypic clustering of Antarctic isolates based on comparisons of physiological and morphological characteristics. Each character was given equal weight, in the analysis comparison was by Burr's Strategy using the software TAXON.

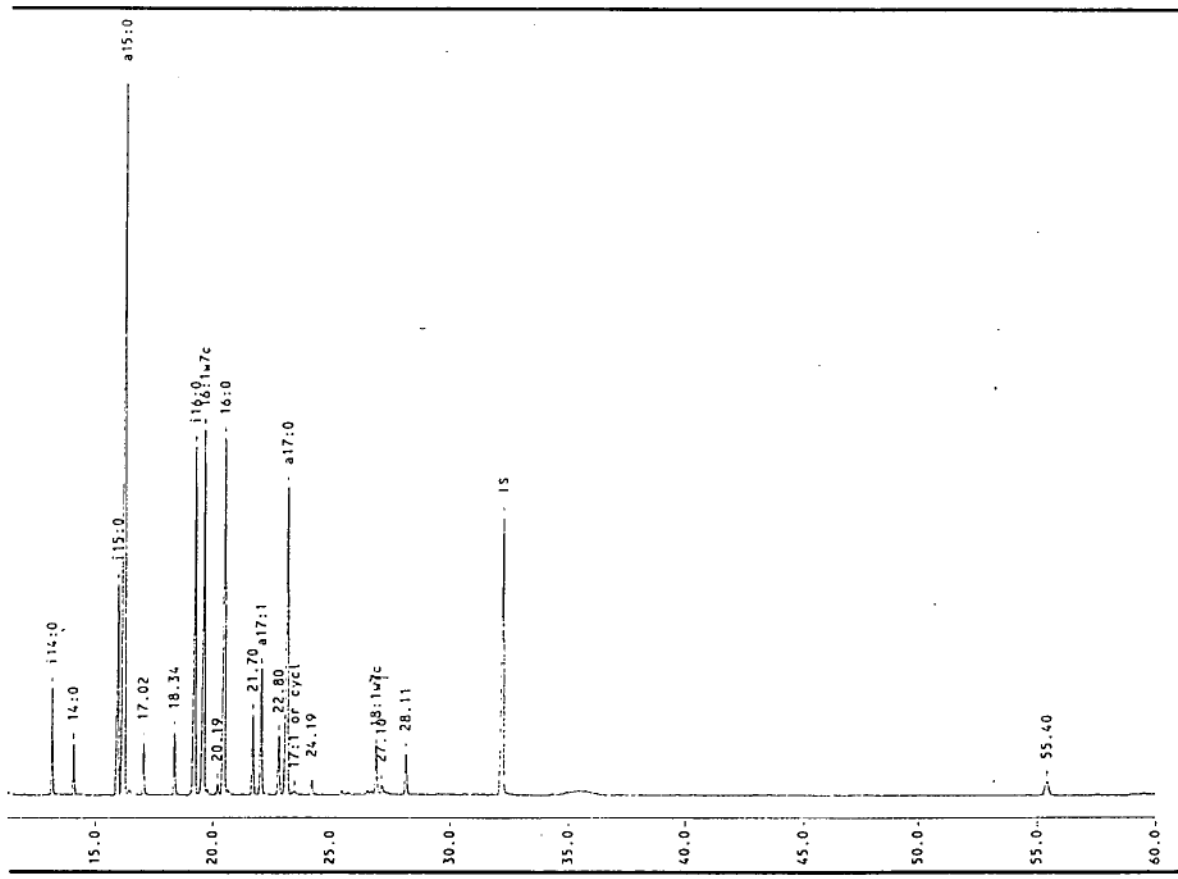


3.6 Chemotaxonomy

3.6.1 Fatty Acids Method 1

Whole cell fatty acids were extracted from each of the Antarctic isolates and the fatty acid composition of each extract was determined by gas chromatography (Table 3.10, Figure 3.2). Major fatty acids of all isolates were ante-iso 15:0 (ranging from 13-35% of total fatty acids but greater than 20% of the total fatty acids in a majority of isolates) and ante-iso 17:0 (ranging from 8-16% of total fatty acids but greater than 10% of the total in a majority of isolates). Iso 16:0, 16:1w7c and 16:0 fatty acids were major components in most isolates, ranging from 5-27%, 3-15% and 5-18% of total fatty acids respectively. A range of other fatty acids were present in all of the isolates as minor components.

Figure 3.2: Fatty acid profile of Antarctic isolate 18a. This profile is typical of those obtained for all Antarctic isolates. Different isolates had different proportions of each of the fatty acids but there were no differences in the types of fatty acids present amongst isolates.



The fatty acid profiles of each of the isolates were compared with one another using the un-standardized average method with SAS software. These data were used to construct a tree showing relative similarity of the fatty acid profiles between all isolates (Figure 3.3).

Five separate cultures of *Streptomyces griseus* (DSM 40236) were grown and the fatty acid profile was determined for each of these cultures. These five *S. griseus* fatty acid profiles were included in the cluster analysis to determine the reproducibility of the fatty acid analyses and in the method of clustering. Each of the five *S. griseus* profiles obtained were clustered together in a well defined group (Figure 3.3) indicating that both reproducibility and method of clustering were reliable. Few of the Antarctic isolates clustered into such well defined groups as the five repeats of *S. griseus* (Table 3.19). However, comparison of the clusters obtained by phenotypic analysis (Figure 3.1) with the relationships between fatty acid profiles (Figure 3.3) showed some similarities. For example, isolates 9a, 9c, 9r, S9a and S9c were clustered together in both numerical taxonomy (Cluster "A") and fatty acid analysis, although isolates 9e, S9e and S9d were included in numerical taxonomy cluster "A" but were not closely clustered to isolates 9a, 9c, 9r, S9a and S9c in the fatty acid analysis.

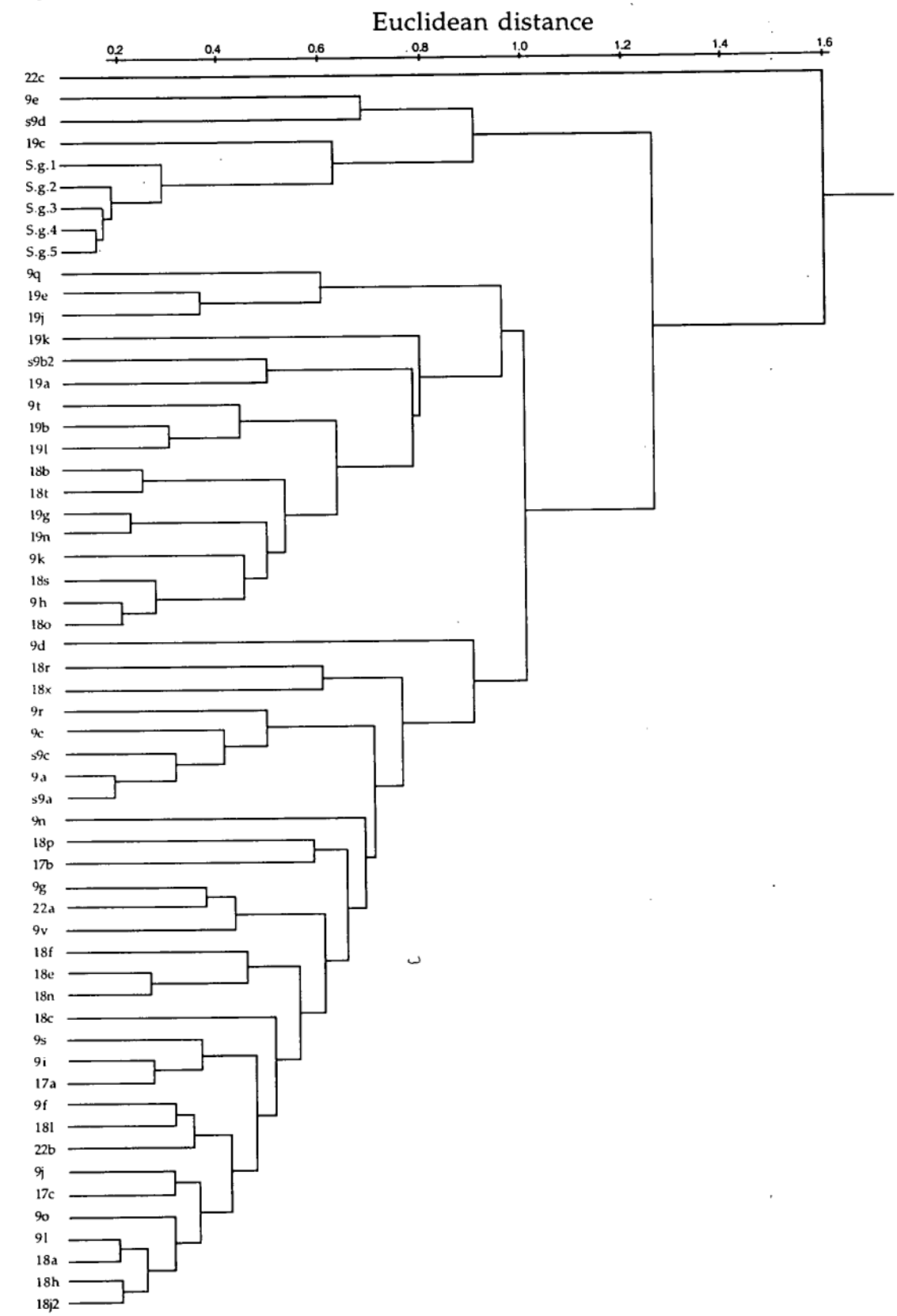
Table 3.10: Menaquinone and Fatty acids from Antarctic Actinomycete strains. Major menaquinone peaks: ++++ = main peak, +++ = any peak > 50% of main peak, ++ = any peak > 25% and <50% of main peak, + = any other >1% of main peak. Fatty acids are shown as a percentage of total fatty acids, rounded to the nearest whole number, t = < 0.5%. "sgr "= *Streptomyces griseus.*

	Menaquinones					Fatty Acids																		
Strain No.	8(H) 6	9(H) 2	9(H) 4	9(H) 6	9(H) 8	i14:0	14:1	14:0	i15:0	a15: 0	15:0	i16:1	i16:0	16:1 w7c	16:0	i17:1	a17: 1	i17:0	a17: 0	17:1 or cyc	17:0	18:2 w6	18:i w9c	18:i w7c	18:0
9a	+	+	+	++++	+++	1	-	1	5	25	1	1	10	10	14	3	4	3	16	t	t	t	2	1	1
9c	+	+	+	++++	+++	1	-	2	-	24	1	1	9	13	15	4	5	2	16	1	t	-	3	1	2
9r	+	+	+	++++	+++	2	-	2	4	21	1	1	10	14	16	3	4	2	13	t	t	t	4	1	3
22c	-	+	+	++++	+++	1	9	2	3	15	-	1	5	12	16	3	4	2	13	2	-	2	6	1	6
s9a	-	+	+	++++	+++	1	-	2	5	25	1	1	9	11	14	3	4	2	16	1	-	-	3	-	-
s9c	+	+	+	++++	+++	1	-	2	3	25	-	1	9	13	16	2	4	2	16	1	-	-	3	-	2
9d	+	+	+	++++	+++	2	-	2	11	22	1	1	7	13	17	4	2	4	10	1	t	-	2	1	1
9e	+	+	+++	++++	+	2	-	1	7	13	t	2	19	9	11	6	4	4	14	1	t	-	3	1	2
s9d	-	+	+	++++	+++	4	-	2	12	16	t	2	17	7	12	4	2	5	8	-	-	-	4	1	3
9t	-	+	+++	++++	+	3	-	1	4	29	1	1	15	5	8	2	3	3	19	1	t	1	2	t	1
s9b2	+	+	+	++++	+++	2	4	1	3	25	1	2	13	8	9	2	6	1	14	3	t	t	3	t	2
9f	+	+	++	++++	++	3	-	2	3	29	1	2	13	10	14	1	3	1	10	-	1	-	4	1	3
9g	+	+	++	++++	++	2	2	1	6	24	1	1	11	12	12	3	5	2	11	1	t	t	2	2	t
9h	+	+	+	++++	+++	3	-	1	4	28	1	2	17	10	10	1	3	1	10	1	t	t	2	1	2
9i	+	+	++	++++	+++	1	t	2	5	26	2	1	7	12	17	1	3	2	13	t	1	t	3	1	3

9j	+	+	++	++++	++	1	t	2	5	27	2	1	8	14	14	2	4	1	11	1	t	-	2	1	3
9k	+	+	++	++++	++	4	t	1	6	30	1	1	20	6	9	1	3	1	11	1	t	-	2	-	2
9l	+	+	+	++++	++	2	-	1	6	29	2	2	10	11	14	1	3	1	11	t	1	t	2	1	2
9n	+	+	++	++++	++	1	-	2	4	33	2	1	8	11	10	1	6	1	15	t	t	-	2	1	2
9o	+	+	+	++++	++	1	-	1	5	32	2	1	11	9	12	1	4	1	14	t	1	t	2	t	2
9q	+	+	++	++++	++	4	t	1	3	35	1	3	22	6	7	1	3	1	11	t	t	-	1	-	1
9s	+	+	++	++++	++	1	-	1	5	29	2	1	8	9	15	1	3	2	13	-	1	2	5	1	2
9v	+	+	++	++++	+++	2	-	2	5	24	1	2	9	16	14	2	3	1	9	t	t	2	4	1	3
17a	+	+	++	++++	++	1	-	2	5	28	2	1	9	11	17	1	3	1	11	-	1	-	3	t	3
17b	+	+	++	++++	+	2	-	2	6	29	2	1	7	13	18	1	2	2	8	-	1	-	4	1	3
17c	+	+	++	++++	++	2	-	2	4	29	2	2	10	14	13	1	3	1	10	-	1	-	3	1	2
18a	++	+	+++	++++	++	1	-	1	6	31	2	1	9	10	14	1	4	1	12	-	1	-	2	1	2
18b	+	+	+++	++++	++	2	-	1	6	24	1	1	16	10	10	2	4	2	12	2	t	1	2	1	2
18c	+	+	++	++++	+	1	t	2	5	27	2	2	10	11	14	2	5	1	14	1	-	-	-	4	-
18e	+	+	++	++++	++	2	-	2	-	28	2	2	11	15	15	2	4	1	11	-	1	-	3	t	2
18f	+	+	++	++++	+++	2	-	2	-	30	1	2	12	8	13	2	4	2	14	-	1	-	3	t	3
18h	+	+	++	++++	++	2	-	1	6	30	1	1	11	11	12	2	4	2	13	t	t	1	1	t	2
18j2	-	+	++	++++	+++	2	-	1	7	29	1	2	11	12	12	2	4	2	11	t	t	-	1	t	1
18l	-	+	+	++++	+++	2	-	1	6	27	2	1	11	10	14	2	4	1	11	-	1	-	4	t	3
18n	+	+	++	++++	++	2	-	2	-	28	2	2	11	12	15	1	4	1	11	-	1	-	4	1	3
18o	+	+	++	++++	++	4	-	2	6	29	1	2	17	10	12	1	3	1	9	-	-	-	1	-	2
18p	+	+	++	++++	+++	2	-	3	-	27	2	2	8	17	17	1	3	1	9	t	1	-	4	1	4
18r	+	+	++	++++	+++	3	-	2	-	23	1	3	15	12	13	2	5	1	10	t	1	-	5	1	4

18s	+	+	++	++++	+	3	-	2	5	30	1	2	15	8	12	1	4	1	10	-	t	-	2	1	3
18t	+	+	++	++++	++	2	-	1	5	26	1	2	16	12	11	2	4	1	11	t	t	t	2	t	1
18x	+	+	++	++++	++	3	-	2	7	25	2	2	13	9	15	1	2	1	8	-	1	-	5	1	3
22a	+	++	+	++++	+++	1	4	1	5	27	2	2	9	15	12	1	4	1	11	1	1	t	2	t	2
22b	+	+	+	++++	+++	3	-	2	4	30	1	3	11	11	12	1	3	1	9	1	1	-	3	1	3
19a	-	+	+	++++	+++	2	6	1	5	23	1	4	16	6	6	4	7	1	12	1	-	t	3	-	2
19b	+	+	+	++++	+++	1	-	2	5	28	1	3	13	5	8	3	6	1	16	t	t	-	4	1	3
19c	+	+	+	++++	++	9	-	2	5	20	1	2	27	4	7	3	3	1	9	1	t	-	3	t	2
19e	+	+	++	++++	++	4	-	1	4	31	1	4	23	3	5	2	5	1	12	t	t	-	3	t	1
19g	-	+	+	++++	++	3	-	1	8	28	1	3	18	5	7	3	4	1	11	t	t	-	3	t	2
19j	-	+	+	++++	++	2	-	1	4	28	t	4	23	3	5	2	6	1	16	t	t	t	2	-	2
19k	+	+	+	++++	++	2	6	2	4	30	1	2	12	3	9	1	3	3	14	t	1	t	4	1	5
19l	+	+	+	++++	+	2	-	1	6	31	1	3	13	5	8	3	6	2	14	-	-	-	3	1	2
19n	+	+	+	++++	++	3	-	2	9	26	1	3	17	5	9	3	4	1	11	-	-	-	4	1	3
sgra	+	+	+	++++	+++	7	-	1	8	22	1	2	21	6	9	3	3	3	8	4	1	1	1	1	t
sgrb	-	+	+	++++	+++	8	-	1	9	25	1	2	20	6	8	3	3	2	7	3	t	t	t	t	-
sgrc	-	+	+	++++	+++	7	-	1	8	22	1	2	23	5	9	3	3	3	8	3	1	t	t	1	t
sgrd	+	+	++	++++	+++	7	-	1	9	23	1	2	22	6	9	3	3	3	8	2	1	t	t	t	t
sgre						6	-	1	8	22	1	2	21	6	9	3	3	3	8	2	1	t	2	t	t

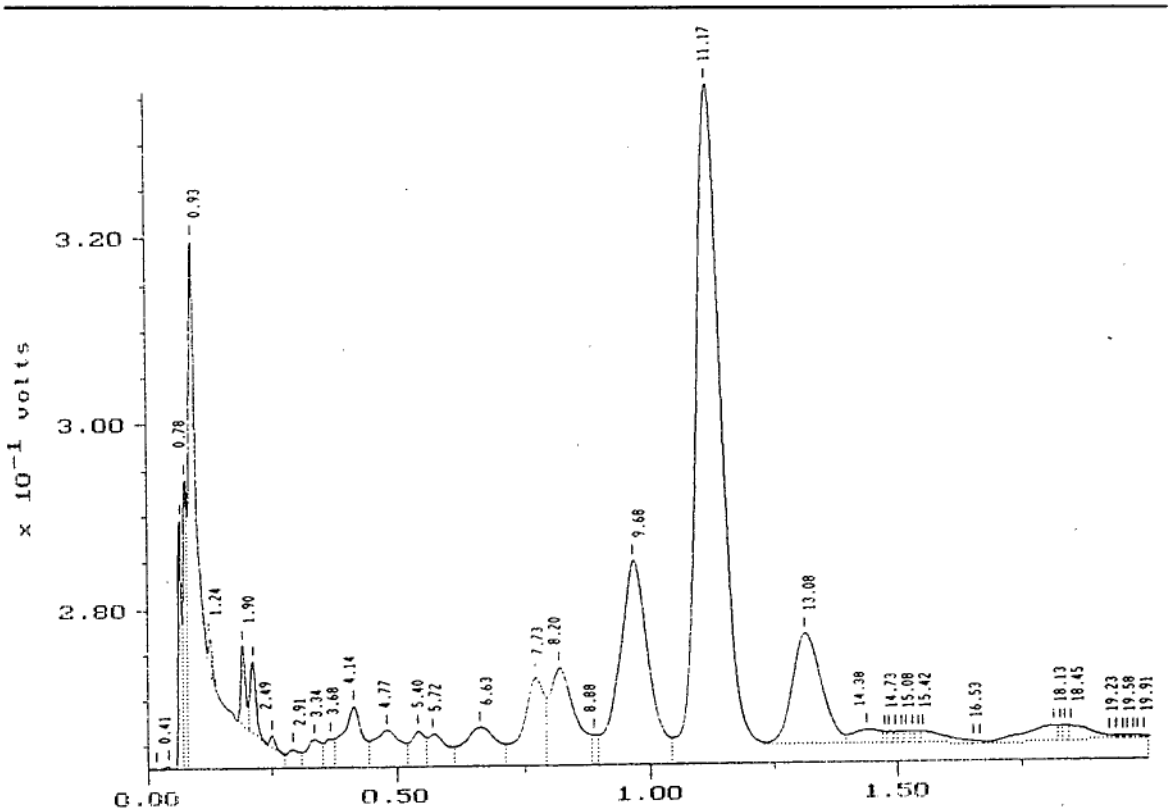
Figure 3.3: Relationships between Antarctic isolates based on comparisons of fatty acid profiles. Fatty acids were extracted and analysed using method one. Fatty acid profiles were compared by SAS cluster analysis using nearest neighbour un-standardized method.



3.6.2 Menaquinones

Menaquinones were extracted from each of the Antarctic isolates and analysed by HPLC (Figure 3.4, Table 3.10). The menaquinone profile of the control strain, *S.griseus* (DSM 40236), matched the profile previously reported for that strain (Korn-Wendisch and Kutzner, 1992). All Antarctic isolates contained 9(H)6 and 9(H)8 as their major respiratory lipoquinones. Menaquinones 9(H)2 and 9(H)4 were also in all isolates, most often as minor components but sometimes in major proportions. Menaquinone 8(H)6 was present as a minor component in many of the isolates. The menaquinone profiles of the Antarctic isolates are consistent with profiles of members of the genus *Streptomyces* (Goodfellow, 1989). The identification of all Antarctic species as belonging to the genus *Streptomyces* was confirmed by the menaquinone analysis in conjunction with fatty acid (Table 3.10) and morphological data (Goodfellow, 1989; Kroppenstedt, 1985).

Figure 3.4: HPLC Menaquinone profile of Antarctic isolate 18a. This profile is typical of those obtained for each of the Antarctic isolates. Peaks were identified by comparison with standard curves which were constructed using elution times of menaquinones extracted from organisms with known menaquinone profiles (Appendix A.2)



3.6.3 Fatty Acids Method 2

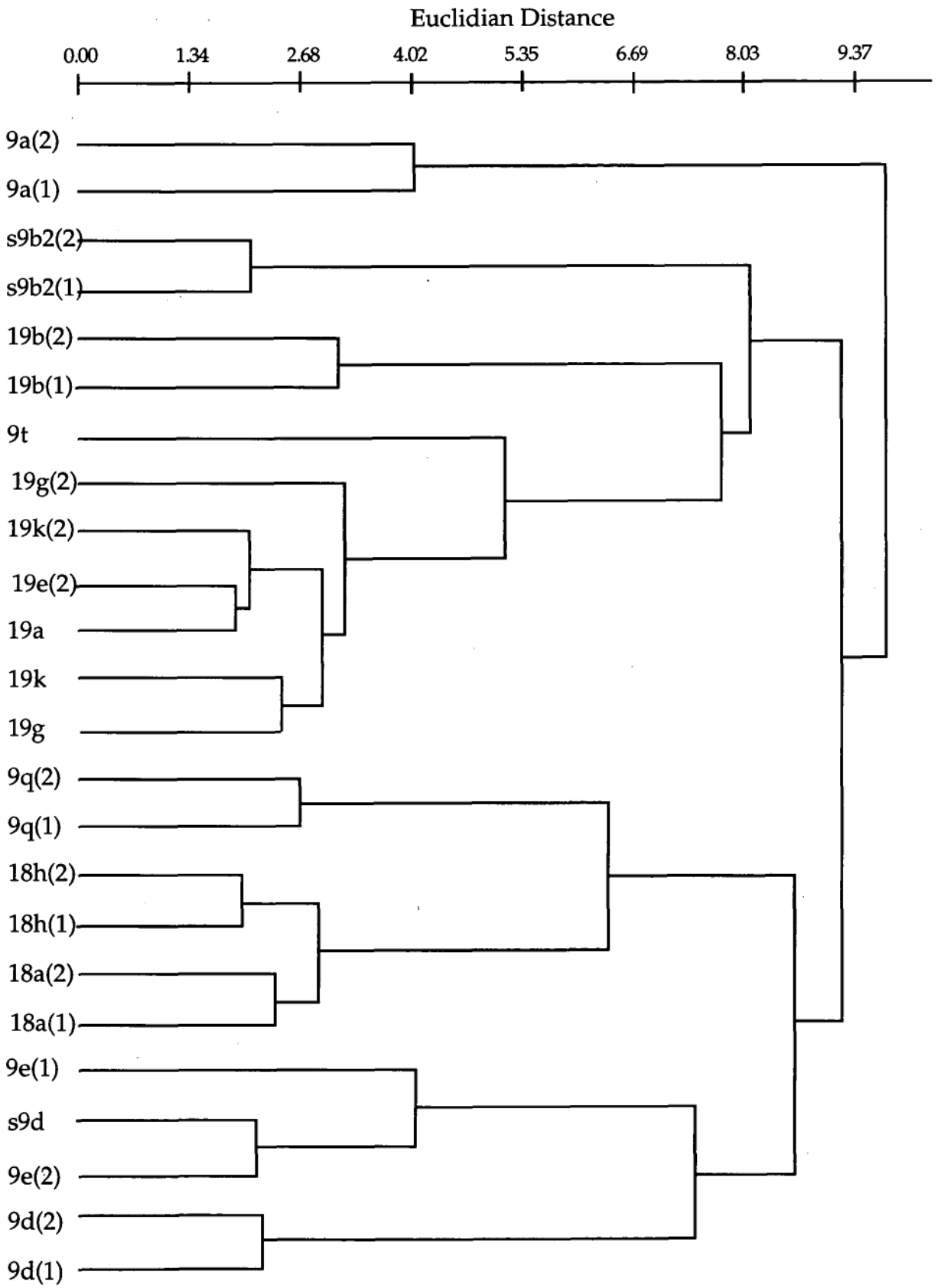
Whole cell fatty acids were extracted and analysed by gas chromatography using a different method from that of 3.6.1 above (these two different methods are given in Section 2.8.1). The fatty acids in the second method were isolated and analysed in the Deutche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and had the advantage that the profiles could be compared with the DSMZ database of actinomycete fatty acid profiles. Fatty acid profiles were determined for each of 14 Antarctic isolates using method two (Table 3.11).

Fatty acid profiles were compared using the single link un-standardized method on SAS software and this data was used to construct a tree showing relative similarity of the fatty acid profiles of each isolate (Figure 3.5).

Table 3.11: Fatty acid composition of 14 Antarctic strains. These fatty acids were extracted using method two (Section 2.8.1). Values are given as percentage of the total fatty acids, "t" indicates trace amounts. Some strains were analysed in duplicate, one of which is followed by the numeral "2" in parentheses

Strain No.	Fatty Acids																			
	i14-0	14:0 0	i15:0	a15:0	15:1	15:0	i16:1	i16:0	16:1 w7c	16:0	16:0 meth-yl	a17:1	i17:0	a17:0	17:1	17:0 cyclo	17:0	17:0 meth-yl	i18:1	18:0
9a	3		4	26			2	13	8	8	3	7	2	22		3				
9a(2)	1	t	4	25		1	2	14	8	8	3	7	2	18	1	4		1	1	
9d	2		11	18		1	3	15	7	5	8	6	4	12	1	4		1	1	
9d(2)	2	t	10	18		1	4	15	7	4	8	6	3	11	1	5		1	1	
9e	3		8	19			5	21	9	7	5	5	4	12		2				
9e(2)	2	t	8	20		2	4	20	8	6	5	5	3	12	2	2		1	1	
s9d(2)	3	t	8	20		3	4	19	8	6	5	5	3	11	2	2	t	1	1	
9t	4		5	27		1	5	19	5	4	3	5	3	13	1	3		1	2	
s9b2	7		4	22			8	25	3	3	2	6	2	10		2		1	2	
s9b2(2)	7		5	23			8	24	3	4	2	5	2	10		3			2	
9q	3	1	11	30		1	5	9	12	5	5	7	2	6	1	3				
9q(2)	2	1	9	31		1	4	9	12	5	4	8	1	8	1	3				
18a	2	t	10	23		1	6	14	11	6	5	7	2	8	1	2			1	
18a(2)	2		10	24		1	6	13	10	6	5	9	2	9	1	2			1	
18h	2	t	9	26		2	5	13	10	6	4	8	2	8	1	2			1	
18h(2)	2	t	10	26		2	6	13	10	5	4	7	2	7	1	2			1	
19a	3		5	28		2	3	21	4	2	4	6	2	15	2	2				
19b	1		7	33		1	1	11	6	4	5	7	2	16		2				1
19b(2)	2		7	31		1	1	12	5	3	5	7	2	17	1	2				
19e(2)	3		5	29	1	2	3	20	4	2	4	6	2	15	2	2			1	
19g	3		5	32	1	2	3	19	3	2	3	6	2	15	2	1				
19g(2)	3		6	29	1	2	3	18	4	2	4	5	3	14	2	2				
19k	3		5	30	1	2	2	19	4	2	3	6	2	16	1	2			t	
19k(2)	3		5	30	1	2	3	21	3	2	3	6	2	14	1	1	t		t	

Figure 3.5: Relationships between Antarctic isolates based on fatty acid profiles, determined by method two. Profiles of some isolates were prepared from duplicate cultures, which is indicated by the numbers in parentheses.



There were some similarities in clustering patterns of the 14 isolates investigated using fatty acid method two when compared with the results obtained from phylogenetic analysis and cluster analysis of fatty acids profiles obtained through method one. However, as with the cluster analysis in fatty acid method one, the cluster analysis in method two did not give well defined groups. There was one obvious difference between the results from the phylogenetic analysis and fatty acid cluster analysis results. Phylogenetic analysis cluster "A" comprised of strains 9a, 9c, 9d, 9e, 9r, S9a, S9e, and S9d but fatty acid analysis placed strains 9d, 9e and S9d into a separate group.

Fatty acid profiles obtained using method two were compared to the profiles contained in the DSMZ database. This comparison gave a number of matches from the database for each of the test organisms but none of the Antarctic isolates could be identified within the confidence limits of this method (Table 3.12). These results indicated that all Antarctic strains were different from those with fatty acid profiles in the DSMZ database.

Table 3.12: Closest match between fatty acid profiles of Antarctic strains and fatty acid profiles of actinomycetes contained in the DSMZ database. Clusters refer to those of Kampfer *et al.*, (1991). Species belonging to the same cluster will generally have a relative similarity of greater than 0.7. A score of 1.0 = equivalent profiles.

Antarctic Strain	Similarity to	Reference Strain	Kampfer Cluster
S9d	0.349	*S. lavendulae*	22-3
9d(2) and 9d	0.059 and 0.070	*S. thermoviolaceus*	4 excl. *S. graminofaciens*
	0.057 and 0.117	*S. rochei glaucescens*	6
	0.042	*S. lavendulae*	22-3
9e and 9e(2)	0.476 and 0.392	*S. lavendulae*	22-3
9a and 9a(2)	0.164 and 0.221	*S. thermoviolaceus*	4 excl. *S. graminofaciens*
	0.139 and 0.115	*S. violaceus coelescens*	9
	0.101 and 0.158	*S. lavendulae*	22-3
9t	0.301	*S. analatus*	1-3
	0.285	*S. thermoviolaceus*	4 excl. *S. graminofaciens*
	0.204	*S. lavendulae*	22-3
	0.190	*Streptoverticillium*	
S9b2 and S9b2(2)	0.211 and 0.197	*S. analatus*	1-3
	0.122 and 0.113	*S. prasinopilosus*	-
	0.114	*S. fradiae*	22-5
19k(2) and 19k	0.484 and 0.490	*S. thermoviolaceus*	4 excl. *S. graminofaciens*
	0.476 and 0.403	*S. analatus*	1-3
	0.242 and 0.269	*S. griseochromogenes*	1-5
19g and 19g(2)	0.492	*S. analatus*	1-3
	0.410 and 0.482	*S. thermoviolaceus*	4 excl. *S. graminofaciens*
	0.333	*S. analatus*	1-3
19e(2)	0.425	*S. thermoviolaceus*	4 excl. *S. graminofaciens*
	0.341	*S. analatus*	1-3
	0.225	*S. griseochromogenes*	1-5
19b and 19b(2)	0.303 and 0.350	*S. thermoviolaceus*	4 excl. *S. graminofaciens*
	0.173	*S. lavendulae*	22-3
19a	0.22-38	*S. thermoviolaceus*	4 excl. *S. graminofaciens*
	0.371	*S. analatus*	1-3
	0.221	*S. griseochromogenes*	1-5
18h and 18h(2)	0.091 and 0.076	*S. lavendulae*	22-3
	0.071 and 0.055	*S. thermoviolaceus*	4 excl. *S. graminofaciens*
	0.067 and 0.059	*S. somaliensis*	type strain
18 a and 18a(2)	0.065 and 0.065	*S. lavendulae*	22-3
	0.044 and 0.059	*S. thermoviolaceus*	4 excl. *S. graminofaciens*
	0.035 and 0.033	*S. violaceus*	9
9q(2) and 9q	0.045 and 0.025	*S. somaliensis*	type strain
	0.030 and 0.026	*S. thermoviolaceus*	4 excl. *S. graminofaciens*
	0.022	*S. lavendulae*	22-3

3.7 Molecular Taxonomy

3.7.1 16S rRNA Sequencing

Partial 16S rRNA sequences were obtained for five Antarctic isolates which were chosen as representative strains from each of the five groups determined by the phenotypic and chemotaxonomic analysis which subsequently confirmed by 16S-23S rRNA spacer pattern analysis (Section 3.7.3). These representative strains were 9a, 9e, 9t, 18a and 19k (Figure 3.6). Partial sequences were also obtained for a number of other isolates (9d, 9q, 9r, 18t, 19n, 22c, S9b2, S9c and S9d). Each of these further sequences showed 100% sequence homology with either 9a, 9e, 9t, 18a or 19k (Table 3.13). These exact sequence homologies confirmed the grouping of strains obtained through numerical taxonomy and fatty acid comparison and 16S-23S rRNA spacer pattern comparison.

Figure 3.6: Partial 16SrRNA sequences of five *Streptomyces* strains isolated from Antarctic soil. The strains are aligned with the 16S rRNA sequence of *Streptomyces griseus*, subsp. *griseus* from Kim *et al.*, (1991), which is given in blue. Varying bases are shown in red. Three hyper-variable regions, as defined by Stackebrandt *et al.*, (1991), are underlined.

```
Sgr   1  AAGUCGAACGAUGAAGCCUUUCGGGGUGGAUUAGUGGCGAACGGGUGAGUAACACGUGGGCA
9 a                    AAGCCUUUCGGGGUGGAUUAGUGGCGAACGGGUGAGUAACACGUGGGCA
9e                     AAGCCUUUCGGGGUGGAUUAGUGGCGAACGGGUGAGUAACACGUGGGCA
9t                     AAGCCCUUCGGGGUGGAUUAGUGGCGAACGGGUGAGUAACACGUGGGCA
18a                    AAGCCCUUCGGGGUGGAUUAGUGGCGAACGGGUGAGUAACACGUGGGCA
19e                    AAGCCCUUCGGGGUGGAUUAGUGGCGAACGGGUGAGUAACACGUGGGCA

Sgr  61   AUCUGCCCUUCACUCUGGGACAAGCCCUGGAAACGGGGUCUAAUACCGGAUAACACUCUG
9 a        AUCUGCCCUUCACUCUGGGACAAGCCCUGGAAACGGGGUCUAAUACCGGAUAACACUCUG
9e         AUCUGCCCUUCACUCUGGGACAAGCCCUGGAAACGGGGUCUAAUACCGGAUAACACUCUG
9t         AUCUGCCCUUCACUCUGGGACAAGCCCUGGAAACGGGGUCUAAUACCGGAUAACACUCUG
18a        AUCUGCCCUUCACUCUGGGACAAGCCCUGGAAACGGGGUCUAAUACCGGAUAUUACUCCU
19e        AUCUGCCCUUCACUCUGGGACAAGCCCUGGAAACGGGGUCUAAUACCGGAUAACACUCUC

Sgr  121  UCCCGCAUGGGACGGGGUUAAAAGCUCCGGCGGUGAAGGAUGAGCCCGCGGCCUAUCAGC
9 a       UCCUGCAUGGGACGGGGUUAAAAGCUCCGGCGGUGAAGGAUGAGCCCGCGGCCUAUCAGC
9e        UCCCUCAUGGGGCGGGGUUAAAAGCUCCGGCGGUGAAGGAUGAGCCCGCGGCCUAUCAGC
9t        UCCCGCAUGGGACGGGGUUAAAAGCUCCGGCGGUGAAGGAUGAGCCCGCGGCCUAUCAGC
18a       GCCUGCAUGGACGGGGGUUAAAAGCUCCGUCGGUGAAGGAUGAGCCCGCGGCCUAUCAGC
19e       CCCCUCCUGGGGGUUGGUUGAAAGCUCCGGCGGUGAAGGAUGAGCCCGCGGCCUAUCAGC

Sgr  181  UUGUUGGUGGGGUAAUGGCCUACCAAGGCGACGACGGGUAGCCGGCCUGAGAGGGCGACC
9 a       UUGUUGGUGGGGUAAUGGCCUACCAAGGCGACGACGGGUAGCCGGCCUGAGAGGGCGACC
9e        UUGUUGGUGGGGUAAUGGCCUACCAAGGCGACGACGGGUAGCCGGCCUGAGAGGGCGACC
9t        UUGUUGGUGGGGUAAUGGCCUACCAAGGCGACGACGGGUAGCCGGCCUGAGAGGGCGACC
18a       UUGUUGGUGGGGUAAUGGCCCACCAAGGCGACGACGGGUAGCCGGCCUGAGAGGGCGACC
19e       UUGUUGGUGGGGUAAUGGCCUACCAAGGCGACGACGGGUAGCCGGCCUGAGAGGGCGACC
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Sgr  241  GGCCACACUGGGACUGAGACACGGCCCAGACUCCUACGGGAGGCAGCAGUGGGGAAUAUU
9a         GGCCACACUGGGACUGAGACACGGCCCAGACUCCUACGGGAGGCAGCAGUGGGGAAUAUU
9e         GGCCACACUGGGACUGAGACACGGCCCAGACUCCUACGGGAGGCAGCAGUGGGGAAUAUU
9t         GGCCACACUGGGACUGAGACACGGCCCAGACUCCUACGGGAGGCAGCAGUGGGGAAUAUU
18a        GGCCACACUGGGACUGAGACACGGCCCAGACUCCUACGGGAGGCAGCAGUGGGGAAUAUU
19k        GGCCACACUGGGACUGAGACACGGCCCAGACUCCUACGGGAGGCAGCAGUGGGGAAUAUU

Sgr  301  GCACAAUGGGCGAAAGCCUGAUGCAGCGACGCCGCGUGAGGGAUGACGGCCUUCGGGUUG
9a        GCACAAUGGGCGCAAGCCUGAUGCAGCGACGCCGCGUGAGGGAUGACGGCCUUCGGGUUG
9e        GCACAAUGGGCGCAAGCCUGAUGCAGCGACGCCGCGUGAGGGAUGACGGCCUUCGGGUUG
9t        GCACAAUGGGCGAAAGCCUGAUGCAGCGACGCCGCGUGAGGGAUGACGGCCUUCGGGUUG
18a       GCACAAUGGGCGAAAGCCUGAUGCAGCGACGCCGCGUGAGGGAUGACGGCCUUCGGGUUG
19k       GCACAAUGGGCGCAAGCCUGAUGCAGCGACGCCGCGUGAGGGAUGACGGCCUUCGGGUUG

Sgr  361  UAAACCUCUUUCAGCAGGGAAGAAGCGAGAGUGACGGUACCUGCAGAAGAAGCGCCGGCU
9a         UAAACCUCUUUCAGCAGGGAAGAAGCGCAAGUGACGGUACCUGCAGAAGAAGC
9e         UAAACCUCUUUCAGCAGGGAAGAAGCGCAAGUGACGGUACCUGCAGAAGAAGC
9t         UAAACCUCUUUCAGCAGGGAAGAAGCGCAAGUGACGGUACCUGCAGAAGAAGC
18a        UAAACCUCUUUCAGCAGGGAAGAAGCGCAAGUGACGGUACCUGCAGAAGAAGC
19e        UAAACCUCUUUCAGCAGGGAAGAAGCGCAAGUGACGGUACCUGCAGAAGAAGC

Sgr  421  AACUACGUGCCAGCAGCCGCGGUAAUACGUAGGGCGCAAGCGUUGUCCGGAAUUAUUGGG

Sgr  481  CGUAAAGAGCUCGUAGGCGGCUUGUCACGUCGGAUGUGAAAGCCCGGGGCUUAACCCCGG

Sgr  541  GUCUGCAUUCGAUACGGGCUAGCUAGAGUGUGGUAGGGGAGAUCGGAAUUCCUGGUGUAG

Sgr  601  CGGUGAAAUGCGCAGAUAUCAGGAGGAACACCGGUGGCGAAGGCGGAUCUCUGGGCCAUU

Sgr  661  ACUGACGCUGAGGAGCGAAAGCGUGGGGAGCGAACAGGAUUAGAUACCCUGGUAGUCCAC

Sgr  721  GCCGUAAACGUUGGGAACUAGGUGUUGGCGACAUUCCACGUCGUCGGUGCCGCAGCUAAC
9a                                                          GCCGCAGCUAAC
9e                                                          GCCGCAGCUAAC
9t                                                          GCCGCAGCUAAC
18a                                                         GCCGCAGCUAAC
19k                                                         GCCGCAGCUAAC

Sgr  781  GCAUUAAGUUCCCCGCCUGGGGAGUACGGCCGCAAGGCUAAAACUCAAAGGAAUUGACGG
9a         GCAUUAAGUUCCCCGCCUGGGGAGUACGGCCGCAAGGCUAAAACUCAAAGGAAUUGACGG
9e         GCAUUAAGUUCCCCGCCUGGGGAGUACGGCCGCAAGGCUAAAACUCAAAGGAAUUGACGG
9t         GCAUUAAGUUCCCCGCCUGGGGAGUACGGCCGCAAGGCUAAAACUCAAAGGAAUUGACGG
18a        GCAUUAAGUUCCCCGCCUGGGGAGUACGGCCGCAAGGCUAAAACUCAAAGGAAUUGACGG
19e        GCAUUAAGUUCCCCGCCUGGGGAGUACGGCCGCAAGGCUAAAACUCAAAGGAAUUGACGG

Sgr  841  GGGCCCGCACAAGCAGCGGAGCAUGUGGCUUAAUUCGACGCAACGCGAAGAACCUUACCA
9a         GGGCCCGCACAAGCAGCGGAGCAUGUGGCUUAAUUCGACGCAACGCGAAGAACCUUACCA
9e         GGGCCCGCACAAGCAGCGGAGCAUGUGGCUUAAUUCGACGCAACGCGAAGAACCUUACCA
9t         GGGCCCGCACAAGCAGCGGAGCAUGUGGCUUAAUUCGACGCAACGCGAAGAACCUUACCA
18a        GGGCCCGCACAAGCAGCGGAGCAUGUGGCUUAAUUCGACGCAACGCGAAGAACCUUACCA
19e        GGGCCCGCACAAGCAGCGGAGCAUGUGGCUUAAUUCGACGCAACGCGAAGAACCUUACCA

Sgr  901  AGGCUUGACAUAUACCGGAAAGCAUCAGAGAUGGUGCCCCCCUUGUGGUCGGUAUACAGG
9a        AGGCUUGACAUAUACCGGAAAGCAUCAGAGAUGGUGCCCCCCUUGUGGUCGGUAUACAGG
9e        AGGCUUGACAUAUACCGGAAAGCAUCAGAGAUGGUGCCCCCCUUGUGGUCGGUAUACAGG
9t        AGGCUUGACAUAUACCGGAAAGCAUCAGAGAUGGUGCCCCCCUUGUGGUCGGUAUACAGG
18a       AGGCUUGACAUAUACCGGAAAGCAUUAGAGAUGGUGCCCCCCUUGUGGUCGGUAUACAGG
19k       AGGCUUGACAUACACCGGAAACGCCUAGAGAUAGGCGCCCCCCUUGUGGUCGGUGUACAGG

Sgr  961  UGGUGCAUGGCUGUCGUCAGCUCGUGUCGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCG
9a         UGGUGCAUGGCUGUCGUCAGCUCGUGUCGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCG
9e         UGGUGCAUGGCUGUCGUCAGCUCGUGUCGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCG
9t         UGGUGCAUGGCUGUCGUCAGCUCGUGUCGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCG
18a        UGGUGCAUGGCUGUCGUCAGCUCGUGUCGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCG
19k        UGGUGCAUGGCUGUCGUCAGCUCGUGUCGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCG
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Sgr1021  CAACCCUUGUUCUGUGUUGCCAGCAUGCC UUCGGGGUGAUGGGGACUCACAGGAGACUGC
9 a        CAACCCUUGUUCUGUGUUGCCAGCAUGCCCUUCGGGGUGAUGGGGACUCACAGGAGACUGC
9e         CAACCCUUGUUCUGUGUUGCCAGCAUGCCCUUCGGGGUGAUGGGGACUCACAGGAGACUGC
9t         CAACCCUUGUUCUGUGUUGCCAGCAUGCCCUUCGGGGUGAUGGGGACUCACAGGAGACUGC
18a        CAACCCUUGUUCUGUGUUGCCAGCAUGCCCUUCGGGGUGAUGGGGACUCACAGGAGACCGC
19e        CAACCCUUGUUCUGUGUUGCCAGCAUGCCCUUCGGGGUGAUGGGGACUCACAGGAGACCGC

Sgr1081  CGGGGUCAACUCGGAGGAAGGUGGGGACGACGUCAAGUCAUCAUGCCCCUUAUGUCUUGG
9 a        CGGGGUCAACUCGGAGGAAGGUGGGGACGACGUCAAGUCAUCAUGCCCCUUAUGUCUUGG
9e         CGGGGUCAACUCGGAGGAAGGUGGGGACGACGUCAAGUCAUCAUGCCCCUUAUGUCUUGG
9t         CGGGGUCAACUCGGAGGAAGGUGGGGACGACGUCAAGUCAUCAUGCCCCUUAUGUCUUGG
18a        CGGGGUCAACUCGGAGGAAGGUGGGGACGACGUCAAGUCAUCAUGCCCCUUAUGUCUUGG
19e        CGGGGUCAACUCGGAGGAAGGUGGGGACGACGUCAAGUCAUCAUGCCCCUUAUGUCUUGG

Sgr1141  GCUGCACACGUGCUACAAUGGCCGGUACAAUGAGCUGCGAUGCGCGAGGCGGAGCGAAUC
9 a        GCUGCACACGUGCUACAAUGGCCGGUACAAUGAGCUGCGAUGCGCGAGGCGGAGCGAAUC
9e         GCUGCACACGUGCUACAAUGGCCGGUACAAUGAGCUGCGAUACGCAAGGUGGAGCGAAUC
99t        GCUGCACACGUGCUACAAUGGCCGGUACAAUGAGCUGCGAUGCGCGAGGCGGAGCGAAUC
18a        GCUGCACACGUGCUACAAUGGCCGGUACAAUGAGCUGCGAUACGCAAGGUGGAGCGAAUC
19e        GCUGCACACGUGCUACAAUGGUCGGUACAAUGAGCUGCGAUGCGCGAGGCGGAGCGAAUC

Sgr1201  UCAAAAAGCCGGUCUCAGUUCGGAUUGGGGUCUGCAACUCGACCCCAUGAAGUCGGAGUU
9A         UCAAAAAGCCGGUCUCAGUUCGGAUUGGGGUCUGCAACUCGACCCCAUGAAGUCGGAGUU
9e         UCAAAAAGCCGGUCUCAGUUCGGAUUGGGGUCUGCAACUCGACCCCGUGAAGUCGGAGUU
9t         UCAAAAAGCCGGUCUCAGUUCGGAUUGGGGUCUGCAACUCGACCCCAUGAAGUCGGAGUU
18a        UCAAAAAGCCGGUCUCAGUUCGGAUUGGGGUCUGCAACUCGACCCCAUGAAGUCGGAGUU
19k        UCAAAAAGCCGGUCACAGUUCGGAUUGGGGUCUGCAANUCGACCCCAUGAAGUCGGAGUU

Sgr1261  GCUAGUAAUCGCAGAUCAGCAUUGCUGCGGUGAAUACGUUCCCGGGCCUUGUACACACCG
9 a        GCUAGUAAUCGCAGAUCAGCAUUGCUGCGGUGAAUACGUCCCCGGGCCUUGUACACACCG
9e         GCUAGUAAUCGCAGAUCAGCAUUGCUGCGGUGAAUACGUCCCCGGGCCUUGUACACACCG
9t         GCUAGUAAUCGCAGAUCAGCAUUGCUGCGGUNNAUACGUCCCCGGGCCUUGUACACACCG
18a        GCUAGUAAUCGCAGAUCAGCAUUGCUGCGGUGAAUACGUCCCCGGGCCUUGUACACACCG
19e        GCUAGUAAUCGCAGAUCAGCAUUGCUGCGGUGAAUACGUCCCCGGGCCUUGUACACACCG

Sgr1321  CCCGUCACGUCACGAAAGUCGGUAACACCCGAAGCCGGUGGCCCAACCCC UUGUGGGAGG
9a         CCCGCAACGUCACGAAAGUCGGUAACACCCGAAGCCGGUGGCCCAACCCC UUGUGGGAGG
9e         CCCGCAACGUCACGANAGUCGGUAACACCCGAAGCCGAUGGCCCAACCCGCAANGGGAGGN
9t         CCCGCAACGUCACGAAAGUCGGUAACACCCGAAGCCGGUGGCCCCAACCCCUUGUGGGAGG
18a        CCCGCAACGUCACGAAAGUCGGUAACACCCGAAGCCGGUGGGCCCAACCCNUAAGGGAGGN
19k        CCCGCAACGUCACGAAAGUCGGUAACACCCGAAGCCGGUGGCCCNAACCCCUUGUGGGAGG

Sgr1381  GAGCUGUCGAAGGUGGGACUGGCGAUUGGGACGAAGUCGUAACAAGGUAGCCGUACCGGA

Sgr1441  AGGUGCGGCUGGAUCACCUCCUUUCUAAGGAGCAUCUAGAUUCCGCAAGGAAUCCAGAGC

Sgr1501  CACUACGUCGGCAAAUGUUCGACGG
```
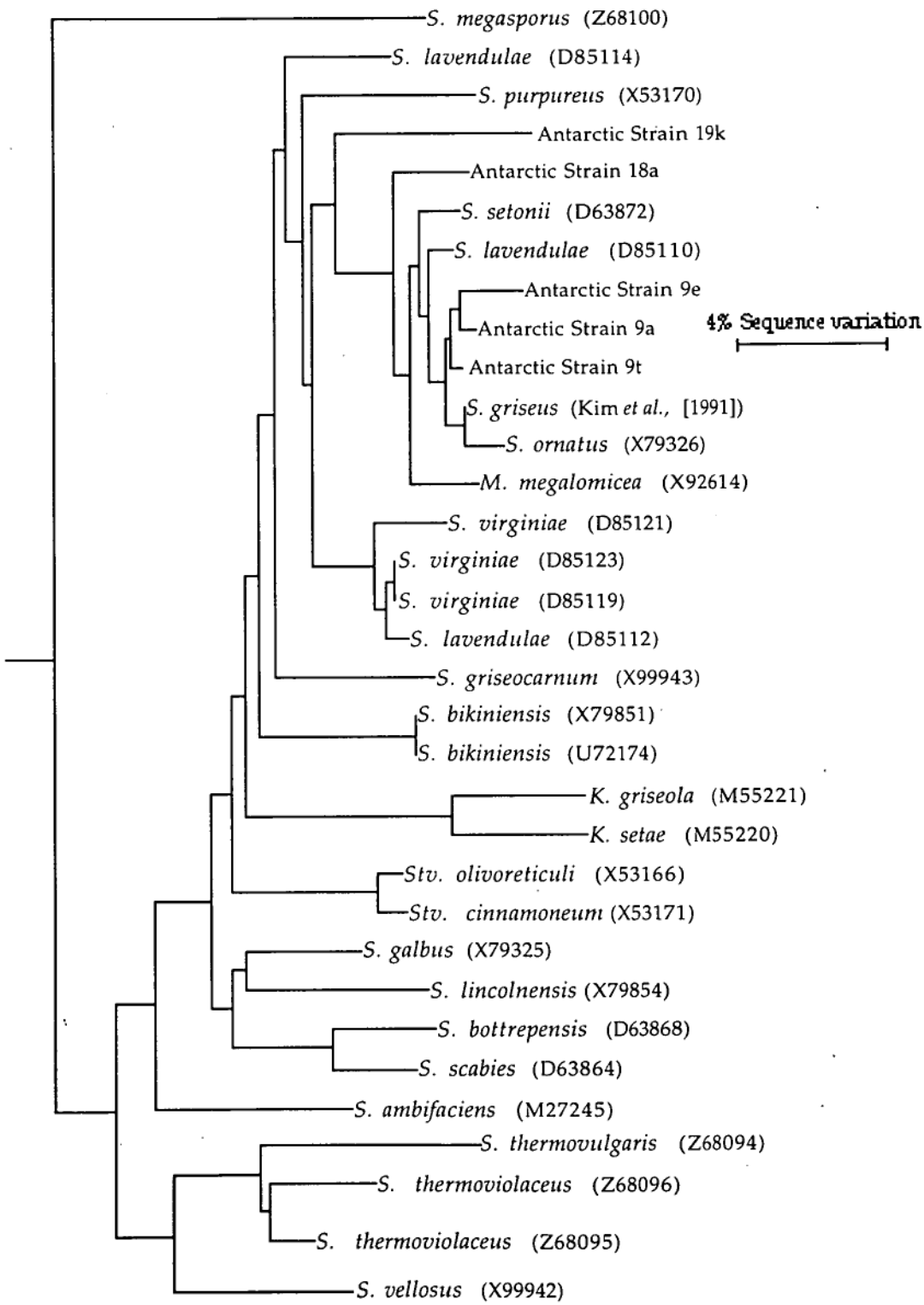

Table 3.13: Partial 16S rRNA sequences of Antarctic isolates 9r, S9c, 22c, 9d, S9d, S9b2, 9q, 18t and 19n were compared with sequences of Antarctic isolates 9a, 9e, 9t, 18a and 19k, whose sequences are given in Figure 3.6. Each sequence in the former group showed 100% homology with one of the sequences in the latter group.

Isolate	Primers used	Isolate showing exact sequence match
9r	530R, Fox	9a
S9c	530R, Fox	9a
22c	530R, 1100F	9a
9d	530R, Fox, 1100F	9e
S9d	530R, Fox	9e
S9b2	530R, Fox, 1100R	9t
9q	530R	18a
18t	1100F, Fox, 530R	18a
19n	530R	19k

Figure 3.7: Relationships between Antarctic isolates and strains in the GenBank database based on 16S RNA sequence comparisons. Sequences were compared by nearest neighbour method using the software package "Phylip" (Felsenstein, 1993). The sequence for each strain used in this comparison was obtained either from GenBank (followed by its GenBank accession number in parentheses) or from the given reference. *Glycomyces harbinensis*, GenBank accession number D85483, was used as the outgroup for this tree. Abbreviations for genera are: *S.=Streptomyces, Stv.=Streptoverticillium, K.=Kitasatosporia, M.=Micromonospora.*



Partial 16S rRNA sequences for five Antarctic isolates, 9a, 9e, 9t, 18a and 19k, were compared to the sequences for all members of the genus *Streptomyces* within the DSMZ sequence database, which included all type strains in the DSMZ culture collection. Seven strains in the database showed a sequence similarity of greater than 99.5% to one or more of the Antarctic isolates (Table 3.14). Some of the strains in the database showed 100% similarity to one another.

Table 3.14: Percentage similarity of partial 16S rRNA sequence with *Streptomyces* strains contained in DSMZ database. Similarity values of equal to or greater than 99.5% are in bold type.

	9e	19k	18a	9a	9t	069	111	128	348	361	236
19k	97.2										
18a	98.0	96.2									
9a	**99.7**	96.9	98.1								
9t	**99.6**	96.8	98.2	**99.9**							
40069	98.0	96.4	99.1	98.1	98.2						
40111	97.8	97.1	96.9	97.8	98.0	97.1					
40128	**99.5**	96.7	98.1	**99.7**	**99.9**	98.3	98.1				
40348	99.0	96.2	97.8	99.2	99.4	98.1	97.6	**99.5**			
40361	**99.5**	96.7	98.1	**99.7**	**99.9**	98.3	98.1	**100**	**99.5**		
40236	99.4	96.6	98.0	**99.6**	**99.7**	98.2	98.0	**99.9**	99.4	**99.9**	
40137	**99.9**	97.1	98.1	**99.6**	**99.7**	98.1	98.0	**99.6**	99.1	**99.6**	**99.5**
40331	**99.5**	96.7	98.1	**99.7**	**99.9**	98.3	98.1	**100**	**99.5**	**100**	**99.9**
40036	98.7	96.4	98.5	98.9	99.0	98.7	97.8	99.1	98.6	99.1	99.0
40163	97.7	96.6	98.3	98.0	98.1	98.6	97.1	98.2	98.0	98.2	98.1
40200	99.2	96.4	97.8	**99.5**	**99.6**	98.1	97.8	**99.7**	99.4	**99.7**	**99.9**
40230	97.8	96.7	98.2	98.1	98.2	98.7	96.8	98.3	98.1	98.3	98.2
40938	99.2	96.6	97.8	**99.5**	99.4	98.1	97.6	**99.5**	99.4	**99.5**	99.4
40257	**99.5**	96.7	98.1	**99.7**	**99.6**	98.3	97.8	**99.7**	**99.5**	**99.7**	**99.6**

40069 = *S. lavendulae*^{T}
40111 = *S. xanthochromogenes*^{T}
40128 = *S. chrysomallus*^{T}
40348 = *S. parvus*^{T}
40361 = *S. analatus*^{T}
40236 = *S. griseus*^{T}
40137 = *S. atroolivaceus*^{T}
40331 = *S. microflavus*^{T}
40036 = *S. spiroverticillatus*^{T}
40163 = *S. lateritius*
40200 = *S. streptomycini*
40230 = *S. venezuelae*^{T}
40938 = *S. floridae*
40257 = *S. vinaceus*

Type strains are marked with a superscript "T"

3.7.2 DNA:DNA Hybridization

DNA:DNA hybridization studies were conducted for Antarctic strains 9a, 9e and 9t and any DSMZ streptomycete with which they showed a 99.5% or greater similarity in partial 16S rDNA sequence (Table 3.14). Hybridization studies were conducted for Antarctic strains 18a and 19k and any DSMZ streptomycete with which they showed a 99% or 97% or greater similarity in partial 16S rDNA sequence (Table 3.14) respectively. Two of the Antarctic strains showed a greater than 70% similarity in DNA:DNA hybridization with DSMZ strains (Table 3.15) and can therefore be accommodated within the same species (Wayne *et al.*, 1987). These strains were 9e with DSMZ 40361 (*Streptomyces analatus*) and 9t with DSMZ 40257 (*Streptomyces vinaceus*) and DSMZ 40200 (*Streptomyces streptomycini*).

Table 3.15: %DNA:DNA hybridization of Antarctic strain DNA with selected *Streptomyces spp.* strains from DSMZ culture collection.

Strain	19k	9e	18a	9a	9t
9e	12				
18a	25	34			
9a	ND	14	ND		
9t	ND	ND	21	33	
DSM 40069	ND	ND	50	ND	ND
DSM 40111	31	ND	ND	ND	ND
DSM 40128	ND	55	ND	37	60
DSM 40348	ND	59	ND	31	58
DSM 40361	ND	72	ND	46	42
DSM 40236	ND	18	ND	37	52
DSM 40137	ND	41	ND	42	12
DSM 40331	ND	30	ND	37	58
DSM 40163	ND	ND	40	ND	ND
DSM 40200	ND	34	ND	38	79
DSM 40938	ND	40	ND	32	42
DSM 40257	ND	44	45	35	75

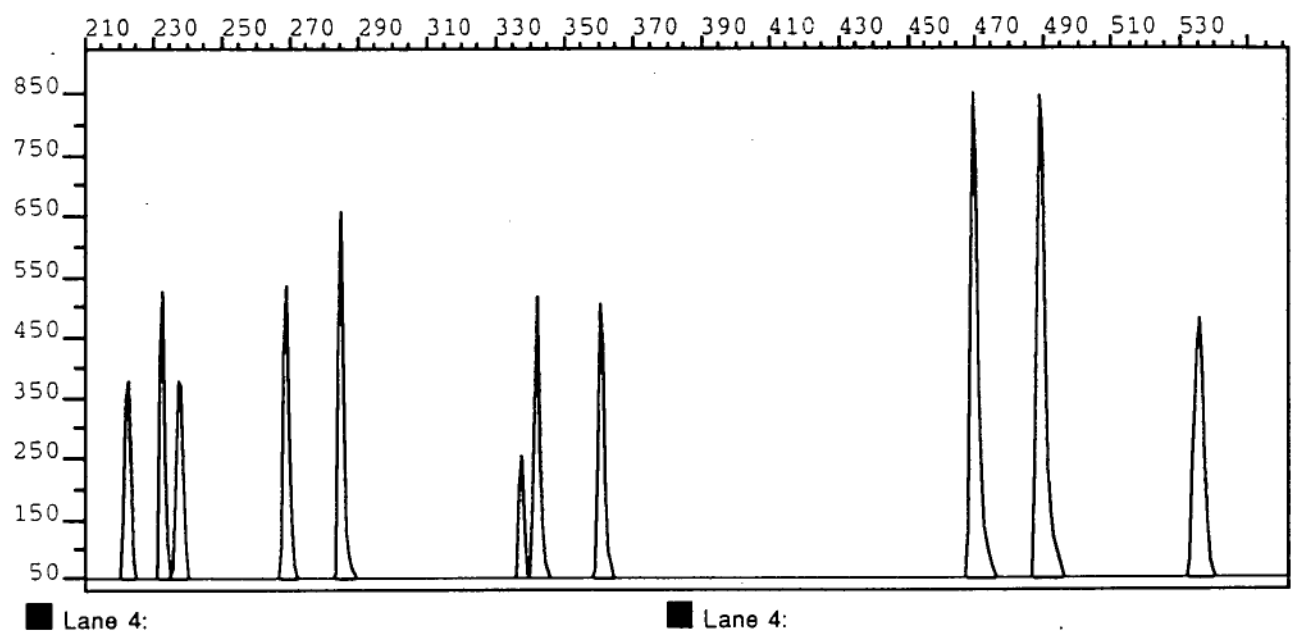
40069 = *S. lavendulae*^{T}
40111 = *S. xanthochromogenes*^{T}
40128 = *S. chrysomallus*^{T}
40348 = *S. parvus*^{T}
40361 = *S. analatus*^{T}
40236 = *S. griseus*^{T}
40137 = *S. atroolivaceus*^{T}
40331 = *S. microflavus*^{T}
40036 = *S. spiroverticillatus*^{T}
40163 = *S. lateritius*
40200 = *S. streptomycini*
40230 = *S. venezuelae*^{T}
40938 = *S. floridae*
40257 = *S. vinaceus*

Type strains are marked with a superscript "T"

3.7.3 16S-23S rRNA Spacer polymorphism

The DNA fragment between the 16S and 23S coding regions was amplified and the size of these fragments determined for selected Antarctic isolates. The isolates were placed into five groups on the basis of these patterns (Table 3.16).

Figure 3.8: 16S-23S rRNA spacer profile for Antarctic isolate S9b2. This profile was of a standard typical of those obtained for all Antarctic isolates.



Peak/Lane	Min.	Size	Peak Height	Peak Area	Scan #
1B, 4	118	338.03	313	2138	1183
2B, 4	119	342.55	601	5131	1199
1R, 4	76	222.00	434	3750	766
2R, 4	80	233.00	588	4141	804
3R, 4	82	238.00	400	4259	822
4R, 4	93	269.00	587	5193	939
5R, 4	99	286.00	745	5846	998
6R, 4	126	361.00	563	4917	1264
7R, 4	162	470.00	934	8761	1620
8R, 4	168	490.00	928	9347	1680
9R, 4	181	536.00	515	6262	1816

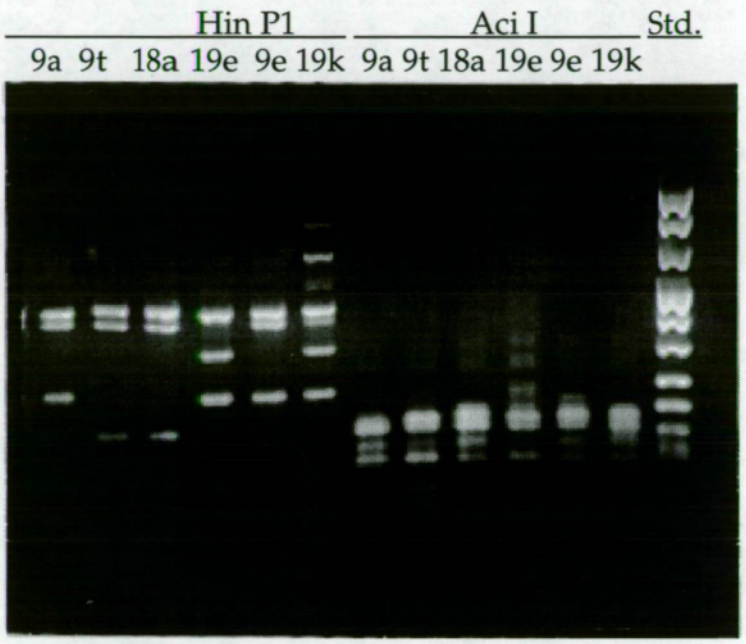
Table 3.16: Grouping of Antarctic strains based on 16S-23S rRNA spacer polymorphism.

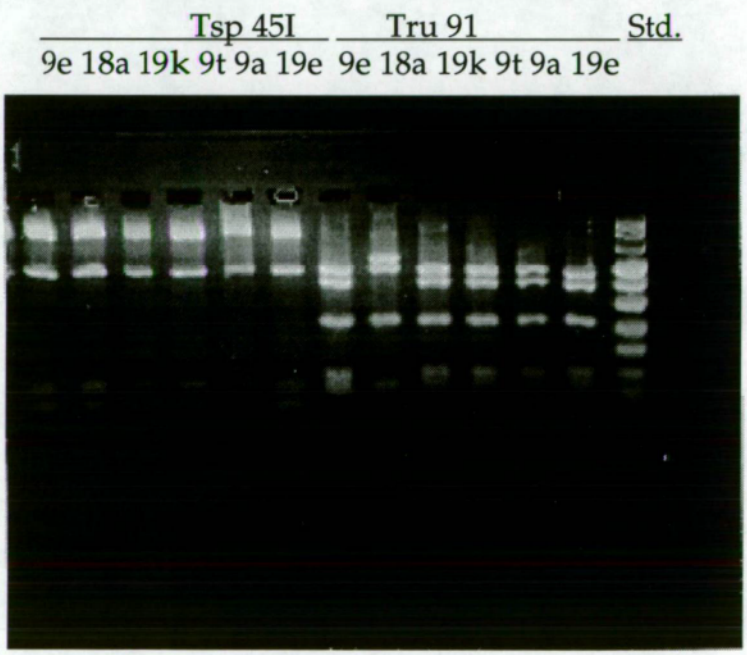
Group	Spacer pattern (Size of fragments given as number of base pairs)		Member isolates
L	337.33 ± 0.440	344.90±0.455	9a, 9c, S9a, S9c, 9r, 22c
M	340.24 ± 0.772	347.94±0.379	9e, 9d, S9d
N	337.33 ± 0.703	342.11±0.558	9t, S9b2
O	323.52 ± 0.539		9f, 9g, 9h, 9i, 9j, 9k, 9l, 9n, 9o, 9q, 9s, 9v, 17a, 17b, 17c, 18a, 18b, 18c, 18e, 18f, 18j, 18l, 18n, 18o, 18p, 18r, 18t, 18x, 22a
P	331.92 ± 0.593		19a, 19b, 19c, 19e, 19g, 19j, 19k, 19l, 19n,

3.7.4 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Sites of known 16S rDNA sequence difference between five Antarctic isolates were compared to the recognition sites for 27 commercially available restriction endonucleases (Table A.3.1, Appendix 3). Based on these criteria four enzymes were selected for use, *Hin* P1 l, *Aci* l, *Tsp* 45 l and *Tru* 9 l.

Figure 3.9: ARDRA patterns produced by restriction enzymes Hin P1, Aci I, Tsp 45 I and Tru 91 after digestion of 16S rDNA of six Antarctic isolates. The standard used in both cases was Boehringer Mannheim molecular weight marker VIII. Molecular weights of bands in standard are (in Kilo-bases, descending): 1114, 900, 692, 501, 489, 404, 320, 242, 190, 147 and 124.





It was expected that banding patterns resulting from ARDRA, using the four enzymes *Hin* P1 I, *Aci* I, *Tsp* 45 I and *Tru* 9 I, would distinguish between each of the five isolates, dividing them into groups as shown in Table 3.17.

Table 3.17: Amplified 16S rDNA restriction analysis of six Antarctic strains. Known differences in 16S rDNA sequence predicted that these Antarctic strains would be placed into groups as shown in the "predicted groups" column on the basis of different banding patterns following enzyme digestion of 16S rDNA and electrophoresis of the digestion product. The actual grouping resulting from each enzyme digestion is given in the right hand column.

Enzyme	Predicted Groups	Groups based on results
Hin P1	(9a, 9e) (9t, 18a) (19k,19e)	(9a, 9e) (9t, 18a) (19k, 19e)
Aci I	(9a, 9e) (9t) (18a, 19k, 19e)	Un-definable*
Tsp 45 I	(9a, 9t, 18a, 19k, 19e) (9e)	No differences in banding
Tru 91	(9a, 9e, 9t, 19k, 19e) (18a)	(9a, 9e, 9t, 19k, 19e) (18a)

*There appeared to be differences in some band intensities but band definition was not sufficient to enable observation of different banding patterns.

However, results were less clear cut than expected (Fig. 3.9, Table 3.17) as only two enzymes produced results which enabled strains to be differentiated. It is possible that optimisation of conditions for Aci I enzyme digestion would have given a better result, but this digestion was performed three times without significant improvement in definition of gel electrophoresis bands. The two enzymes which gave a result placing the Antarctic strains into different groups could between them place the six strains into four groups: (9a, 9e) (9t) (18a) and (19k, 19e).

Chapter 4: Discussion

4.1. Identification of Actinomycetes Isolated from Antarctic Soil

The primary aim of this study was to investigate the diversity of actinomycetes in Antarctic soil and to determine whether there was a population of actinomycetes unique to the Antarctic. In order to achieve this aim it was necessary to identify to species level the 52 hyphal actinomycetes which were isolated from Antarctic soil.

The studies of menaquinone composition, fatty acids and morphology of each of the hyphal actinomycetes isolated from Antarctic soils showed that each isolate was a member of the genus *Streptomyces* (Section 3.6.2). Variations in the 16S-23S rDNA spacer region have been reported to differentiate between strains of the genus *Streptomyces* (Hain, T. O, 1996, personal communication). In the present study, 49 of the 52 Antarctic isolates were placed into 5 groups based on 16S-23SrRNA spacer patterns (Table 3.16). It has been reported that two different strains within the genus *Streptomyces* may share the same 16S-23S rRNA spacer pattern but this occurs rarely (Hain, T. O, 1996, personal communication). Because of this potential for two different strains to share the same 16S-23S rRNA spacer pattern, it was necessary to confirm that each of the five groups of Antarctic isolates based on spacer patterns consisted of similar strains. Data from phenotypic analysis, fatty acid analysis and comparison of 16S rRNA sequences confirmed that each of the five groups based on 16S-23S spacer pattern consisted of similar strains. Three Antarctic isolates, 22b, 18h and 18s, for which 16S-23S spacer pattern data were not available, were placed into one of the groups based on results from numerical taxonomic analysis of phenotypic attributes and fatty acid patterns. These five groups which contained all Antarctic isolates were named "L", "M", "N", "O" and "P" (Table 4.1). A representative isolate was chosen from each of these five taxonomic groups for further study, strains 9a, 9e, 9t, 18a and 19k respectively.

Table 4.1: Placement of Antarctic strains into groups based on all taxonomic data. 16S-23S rDNA spacer patterns can distinguish between members of the genus *Streptomyces* to strain level, so each group is comprised of members which belong to the same species. Representative strains selected from these groups for comparison with the DSMZ actinomycete 16S rDNA sequence database and subsequent DNA:DNA hybridization studies are in bold type. DNA:DNA hybridization studies of representative isolates showed that L, M, N, O and P was each a separate species.

Species	Antarctic strains belonging to this species	Taxonomic data used to place isolates within their designated group
L	**9a**, S9c, 9r, 22c	Phenotypic analysis Fatty acids (method 1) 16S-23S rDNA spacer pattern 16S rDNA sequence comparison
	S9a, 9c	Phenotypic analysis Fatty acids (method 1) 16S-23S rDNA spacer pattern
M	**9e**, S9d	Phenotypic analysis Fatty acids (method 1 and 2) 16S-23S rDNA spacer pattern 16S rDNA sequence comparison
	9d	Phenotypic analysis Fatty acids (method 2) 16S-23S rDNA spacer pattern 16S rDNA sequence comparison
N	**9t**, S9b2	Phenotypic analysis 16S-23S rDNA spacer pattern 16S rDNA sequence comparison
O	**18a**, 9q	Phenotypic analysis Fatty acids (method 2) 16S-23S rDNA spacer pattern 16S rDNA sequence comparison
	18 (h, s) 22b	Phenotypic analysis Fatty acids (Method 1)
	9 (f, g, h, i, j, k, l, n, o, s, v) 17 (a, b, c) 18 (b, c, e, f, j, l, n, o, p, r, t, x) 22a	Phenotypic analysis 16S-23S rDNA spacer pattern
P	**19k**, 19n	Phenotypic analysis 16S-23S rDNA spacer pattern 16S rDNA sequence comparison
	19a, 19e, 19g	Phenotypic analysis Fatty acids (method 2) 16S-23S rDNA spacer pattern
	19b, 19c, 19j, 19l	Phenotypic analysis 16S-23S rDNA spacer pattern

Partial 16S rDNA sequences were determined for the Antarctic isolates chosen as representative strains from groups L, M, N, O and P. The regions included in the partial 16S rDNA sequences included those reported to show greatest variation between different species within the genus *Streptomyces* (Stackebrandt *et al.*, 1991). These highly variable regions were from base 98 to 243, 922 to 938 and 1042 to 1062, using the numbering shown in the present study (Figure 3.6). Variation in 16S rDNA sequences between Antarctic strains occurred in the first two of these variable regions but not in the third. Comparison of 16S rDNA sequences of the five Antarctic isolates, (Figure 3.7, Table 3.14) and subsequent DNA:DNA hybridization results (Table 3.15) showed that each of the five Antarctic isolates, 9a, 9e, 9t, 18a and 19k was a member of a different species. The 16S-23S rRNA spacer patterns placed the 50 Antarctic isolates into five different groups. Therefore, as 16S-23S rRNA spacer patterns can differentiate at strain level, each of the 50 Antarctic isolates belong to one of five species of the genus *Streptomyces.*

Partial 16S rDNA sequences of Antarctic strains 9a, 9e, 9t, 18a and 19k were compared with the partial 16S rDNA sequences of all streptomycetes in the DSMZ sequence database (Figure 3.7). The DSMZ sequence database contains partial 16S rDNA sequences of up to 500 strains. The GenBank database, accessed via the National Centre for Biotechnology Information (NCBI) database (Website: www.ncbi.nlm.nih.gov) contains the 16S rDNA 16S rRNA sequences of 159 streptomycetes available as of the 30th of June, 1997. Some of these 159 streptomycete sequences are duplicates, that were lodged in the database by different investigators. A comparison of the 16S rDNA sequences of Antarctic strains with the sequences available in the NCBI database showed that no 16S rDNA sequence with greater than 99% sequence homology with any Antarctic strains had been deposited in the database. In contrast, nine strains with a sequence similarity of greater than or equal to 99.5% to one or more of the Antarctic strains were found in the DSMZ database (Table 3.14).

In an attempt to define the concept of the term "species" for bacteria, it was proposed by Wayne, *et al.*, (1987) that any two strains of bacteria with a greater than 70% similarity in DNA:DNA hybridization studies would be considered the same species. Fox *et al.*, (1992) suggested that, due to inherent errors in sequencing methods, a 16S rDNA sequence homology of greater than 99% may or may not indicate that two organisms are members of the same species and that relationships between organisms showing such a degree of sequence similarity could only be resolved through DNA:DNA

hybridization. A sequence homology of 97% has been suggested by others (Stackebrandt and Goebel, 1994) as a level beyond which sequence homology becomes unreliable in differentiating between species. However, such a large number of DSMZ strains showed greater than 97% 16S rDNA sequence similarity with Antarctic strains 9a, 9e, 9t and 18a that DNA:DNA hybridisation studies between all DSMZ strains which showed this level of sequence similarity with these four Antarctic strains was impracticable. Even the number of DSMZ strains showing greater than 99% sequence homology with Antarctic strains 9a, 9e and 9t was too great for each DNA:DNA hybridization to be performed.

DNA:DNA hybridization studies were conducted between Antarctic strains 9a, 9e, 9t and 18a and those DSMZ strains with which they showed greater than 99.5% 16S rDNA sequence similarity (Figure 3.7, Table 3.14 and Table 3.15). Antarctic strain 19k was compared to all DSMZ strains with which it had greater than 97% sequence homology as only two DSMZ strains showed sequence homology to this degree. Two Antarctic strains, 9e and 9t, showed greater than 70% similarity in DNA:DNA hybridization with DSMZ strains and can therefore be identified with these DSMZ strains. Antarctic strain 9e had a DNA:DNA hybridization value of greater than 70% with *Streptomyces analatus* (DSMZ type strain 40361) and Antarctic strain 9t, had a DNA:DNA hybridization value of greater than 70% with *Streptomyces vinaceus*(DSMZ strain 40361) and *Streptomyces streptomicini* (DSMZ strain 40200). Antarctic strains 9a, 18a and 19k did not have a hybridization value of greater than 70% with any of the DSMZ strains with which they were compared. Antarctic strain 19k appears to be a novel species of the genus *Streptomyces*. If 99% sequence homology or less is accepted as accurate enough to differentiate between species of *Streptomyces* then strain 18a is a novel species. Antarctic strain 9a could also be a novel species but more DNA:DNA hybridization studies are necessary for clarification.

4.1.1 Description of Antarctic Strains

Strain 9a (representative strain of species L)
On sporulation agar the colour of the spore mass was white, the underside of the colony was orange and no diffusible pigments were produced. Spore chains were retinaculiaperti or reflexibles. Melanin was not produced on PYI agar. Strain 9a utilized arabinose, cellobiose, fructose, galactose, glucose, inulin, mannose, rhamnose, salicin, trehalose and xylose as sole carbon sources. Strain 9a did not produce antibiotics on sporulation agar against any of the test organisms (Section 2.6). Major fatty acids and menaquinones of this isolate are shown in Table 4.2, its partial 16S rRNA sequence is given in Figure 3.6, and its 16S-23S rRNA spacer size in Table 3.16. Other strains in species L varied from 9a in the following characters: Spore chain morphology retinaculiaperti (9c, 9r, S9a, S9c); Spore mass brown (9r); Did not utilize inulin (S9a); utilized *meso*-inositol (S9a, s9c, 22c); utilized lactose (9c, 9r).

Strain 9e (= DSM 40361, *S. analatus*)(representative strain of species M)
On sporulation agar the colour of the aerial mycelium was white, the underside of the colony was translucent and no diffusible pigments were produced. Spore chains were not seen and it is possible that this strain does not produce them. Melanin was produced on PYI agar. Strain 9e utilized arabinose, cellobiose, fructose, galactose, glucose, inulin, mannose, rhamnose, salicin, trehalose and xylose as sole carbon sources. Strain 9e did not produce antibiotics on sporulation agar against any of the test organisms (Section 2.6). Major fatty acids and menaquinones of this isolate are shown in Table 4.2, its partial 16S rRNA sequence is given in Figure 3.6, and its 16S-23S rRNA spacer size in Table 3.16. Other strains in species M varied from 9e in the following characters: Utilized *meso*-inositol (9d, S9d); utilized lactose (9d, S9d).

Strain 9t (= DSM 40257, *S. vinaceus* and DSM 40200 *S. streptomicini*) (representative strain of species N)
On sporulation agar the colour of the spore mass was brown, the underside of the colony was orange or brown and olive coloured diffusible pigment was produced. Spore chains were retinaculiaperti. Melanin was not produced on PYI agar. Strain 9t utilized arabinose, cellobiose, fructose, galactose, glucose, lactose, mannitol, mannose, salicin, trehalose and xylose as sole carbon sources. Strain 9t produced antibiotics that were inhibitory to *Staphylococcus aureus* (ATCC 660) on sporulation agar. Major fatty acids

and menaquinones of this isolate are shown in Table 4.2, its partial 16S rRNA sequence is given in Figure 3.6, and its 16S-23S rRNA spacer size in Table 3.16. Strain S9bs varied from 9a in the following characters: Did not produce antibiotic inhibitory to *Staphylococcus aureus* ; produced antubiotic inhibitory to *Pseudomonas aeruginosa* ; utilized dextran, inulin,*meso*-inositol and rhamnose.

Strain 18a *sp. nov.*(representative strain of species O)

On sporulation agar the colour of the spore mass was violet, the underside of the colony was yellow and brown coloured diffusible pigment was produced. Spore chains were retinaculiaperti or reflexibles. Melanin was not produced on PYI agar. Strain 18a utilized arabinose, cellobiose, fructose, glucose, mannose, salicin, trehalose and xylose as sole carbon sources. Strain 18a produced antibiotics that were inhibitory to *Bacillus subtilis* (Sydney University, Strain 21-6 [Whitehouse]) *Pseudomonas aeruginosa* (ATCC 27853) *Staphylococcus aureus* (ATCC 660) and *Escherichea coli* (Melbourne University, Strain 200) on sporulation agar. Major fatty acids and menaquinones of this isolate are shown in Table 4.2, its partial 16S rRNA sequence is given in Figure 3.6, and its 16S-23S rRNA spacer size in Table 3.16. Other strains in species O varied from 18a in the following characters: Spore mass white (9s, 17b, 18j, 18x, 22b); Spore chain reflexibiles (9k, 9o, 17b, 17c, 18b, 18s); many variations in production of effective antibiotics (see Table 3.5) and carbohydrate utilization (see Table 3.6).

Strain 19k *sp. nov.*(representative strain of species P)

On sporulation agar the colour of the spore mass was grey, the underside of the colony was olive and brown coloured diffusible pigment was produced. Spore chains were spirales. Melanin was produced on PYI agar. Strain 19k utilized arabinose, cellobiose, galactose, glucose, lactose, mannose, melibiose, *meso*-inositol, raffinose, rhamnose, salicin, trehalose and xylose as sole carbon sources. Strain 19k did not produce antibiotics on sporulation agar against any of the test organisms (Section 2.6). Major fatty acids and menaquinones of this isolate are shown in Table 4.2, its partial 16S rRNA sequence is given in Figure 3.6, and its 16S-23S rRNA spacer size in Table 3.16. Other strains in species P varied from 19k in the following characters: Produced antubiotic inhibitory to *Bacillus subtilis* (19a); many variations carbohydrate utilization (see Table 3.6).

Table 4.2: Major fatty acids and menaquinones for five actinomycete strains. Fatty acids, + ≥5% of total fatty acids, ++≥10%, +++≥15%, ++++≥20%, +++++≥25%.

Strain	Major fatty acids							Menaquinones	
	i15:0	a15:0	i16:0	16:1 w7c	16:0	a17:1	a17:0	Major	Minor
9a	-	++++	+	++	+++	-	+++	$9(H)_6$, $9(H)_8$	$9(H)_4$, $9(H)_2$, $8(H)_6$
9e	++	+++	+++	+	++	-	++	$9(H)_6$, $9(H)_8$	$9(H)_4$, $9(H)_2$, $8(H)_6$
9t	-	+++++	++	+	+	-	+++	$9(H)_6$, $9(H)_8$	$9(H)_4$, $9(H)_2$, $8(H)_6$
18a	+	+++++	++	++	++	-	++	$9(H)_6$, $9(H)_8$, $9(H)_4$	$9(H)_2$, $8(H)_6$
19k	+	+++++	+++	+	+	+	+++	$9(H)_6$, $9(H)_8$	$9(H)_4$, $9(H)_2$, $8(H)_6$

4.1.2 Actinomycete Diversity in Antarctic Soils

All actinomycetes isolated from Antarctic soil were members of the genus *Streptomyces* and belonged to five different species. These five species came from soils taken from five different sites. Compared to studies of environments in non-Antarctic climates, where members of a number of different actinomycete genera are commonly isolated from soils and sediments (Takizawa *et al.*, 1993; Xu *et al.*, 1996; Jiang and Xu, 1996), this is a very low level of actinomycete diversity. The actinomycete count in the Antarctic soils was also relatively low. Typically 10^4-10^7 actinomycete colony forming units/g of dry soil are recovered from non-Antarctic soils (Hirsch and McCann-McCormick, 1985), but in Antarctic soils the count ranged from zero to 3.4×10^4 colony forming units/g of dry soil. Counts of actinomycete colony forming units/g of dry soil ranged from 4×10^2 (soil 16) to 3.4×10^2 (soil 18) and the average count was 1.1×10^4 units/g dry soil.

There is good evidence that some species isolated from the Antarctic by previous investigators are indigenous, such as those found in the Antarctic hyper-saline lakes (Dobson *et al.*, 1993; Franzmann *et al.*, 1988a) and soils in the Schirmacher Oasis (Shivaji *et al.*, 1992). Other psychrophilic and psychrotolerant bacteria whose taxonomy is uncertain were also isolated in earlier studies (Benoit and Hall, 1970; Boyd, *et al.*, 1966; Cameron *et al.*, 1970; Horowitz, *et al.*, 1972). Studies across a number of years would be required to establish conclusively that the species obtained in the present study can grow in the Antarctic soil environment. However, four of the five *Streptomyces* species isolated were associated with mosses and lichens

which suggests that growth in Antarctic microbial communities would be possible for these four species. According to Cameron *et al.*, (1970) Antarctic actinomycetes can grow in harsher environments than can Antarctic moss and lichen, so at sites where moss and lichen are found, growth of actinomycetes should be possible. The moss and lichen would provide the actinomycetes with a nutrient source. Liquid water, necessary for the survival of the moss and lichen, must also be present at these sites for part of the year.

Examination of the data indicates that two of the actinomycete species isolated in the present study, species "O" and "P", are novel species. However, of the remaining three species, "M" and "N" were identified as members of previously described species and species "L" appears to be closely related to species "M" and "N" with which its representative strain, 9a, showed a 16S rDNA sequence homology of 99.7% and 99.9% respectively. Therefore the majority of the actinomycete microbiota found in the soil at the margins of the Antarctic continent cannot be considered unique to this environment on this basis.

Members of the genus *Streptomyces* have been isolated from air in the Antarctic dry valleys (Cameron *et al.*, 1972) and pollen from non-Antarctic sources has been found in the moss and lichen beds at the continental margins. Therefore, it is possible that the strains isolated in the present study were wind borne propagules from temperate regions which were culturable, but not growing at the sites from which they were taken. Three out of the five actinomycete species isolated from Antarctic soil were distributed over a number of different sites, with one, species "O" , dominant at four sites. A greater variety of actinomycete species and a more random distribution of propagules would be expected if the actinomycetes only originated as wind borne spores trapped by the moss and lichen.

Antarctic species "M" was identified as *Streptomyces analatus,* and Antarctic species "N" as *S. vinaceus-S. streptomicini.* According to the literature, none of these three previously described species possess any characteristics which appear to make them especially adapted to the Antarctic soil environment (Williams *et al.*, 1983a). All five *Streptomyces* species isolated from the Antarctic grew equally well at 28°C and are therefore not psychrophilic.

It seems unlikely that species ""L", "M", "N" and "O" were anthropogenic contaminants as they were isolated from pristine environments but a human origin for these strains cannot be completely discounted. All members of species "P" (Table 4.1), from which 19k was chosen as a representative, were isolated from a single site which was frequented by humans and their vehicles (Table 3.2). If species "P" was present in soil sample 16 as a result of human contamination, it would seem unusual that only one actinomycete species was isolated from this soil. The volume of soil obtained from site "19" was too small (less than 5ml in volume) to determine whether species "P" was widespread or not in the area used by humans. It is interesting to note that the 16S rDNA sequence of the strain 19k, the representative strain of species "P", showed the lowest degree of homology of any of the five Antarctic species and with any of the streptomycete 16S rDNA sequences in the DSMZ database.

Kerry (1990) reported the presence of fungal contaminants at sites of human disturbance in the Antarctic, but Kerry (1990) also reported a greater variety of fungal species at sites of human disturbance and less species variety in pristine environments. The opposite result was found in the present study of actinomycetes where four species were isolated from pristine environments and one from a site of human disturbance.

The four species of *Streptomyces* isolated from the moss and lichen beds in the Vestfold Hills, "L", "M", "N" and "O", represent the dominant actinomycete microbiota of this environment. The low actinomycete diversity in the soils investigated in the present study supports the findings of Xu *et al.*, (1996) who reported a decrease in the number of actinomycete genera recovered from soils as temperature decreased in the sites from which soil samples were taken. The results in the present study also support those of Xu *et al.*, (1996) who reported a correlation between actinomycete and vegetation diversity. Vincent, (1988) reported that lower numbers of cyanobacterial genera were present in Antarctic soils than in temperate soils, indicating that this trend in the Antarctic soil microbiota is not restricted to the actinomycetes. Although evidence from this study suggests that actinomycetes belonging to non-streptomycete genera would be few, recovery of many "rare" actinomycetes require targeted isolation methods even from environments where they are comparitively abundant. Therefore, such targeted studies would be required before conclusions about the presence of members of rarer actinomycete genera in Antarctic soil could be drawn.

Vishniac and Hempfling (1979) suggested that a lack of appropriate isolation techniques was the main hindrance to a proper understanding of microbial communities in the Antarctic. The discovery, using direct molecular methods, of numerous new species in a well studied environment (Ward *et al.*, 1990) indicated that this problem is not restricted to the Antarctic environment. Actinomycetes can be difficult to isolate as they are slow growing, with lag periods of up to three months for some strains (Benoit and Hall, 1970) so direct molecular detection could be a valuable method for the determination of actinomycete composition in soil communities. However, use of molecular techniques to study Antarctic soil communities could provide misleading data due to the presence in the soil of wind deposited lyophilized material in which DNA is probably preserved by the cold Antarctic environment. Application of direct molecular techniques may be more appropriate in some "enclosed" Antarctic terrestrial environments such as the crypto-endolithic environment.

Cameron *et al.*, (1976) gave a summary of species belonging to the genus *Streptomyces* which had been identified previous to 1976. These species were *Streptomyces albus*, *S. exfoliatus*, *S. longisporoflavus*, and *S. parvus*. None of the species identified by Cameron *et al.*, (1976) were isolated in the present study. This suggests that sampling of a wider variety of sites in the Antarctic may show a wider diversity of actinomycetes than has been found so far.

4.1.3 Biotechnological Potential of the Antarctic Actinomycete Microbiota

Anti-bacterial compounds were produced by Antarctic strains 18a and 9t. The compound produced by strain 18a was identified as gilvocarcin (Dragar, 1993, personal communication) which is a member of the C-glycosides class of antibiotics (Carver, 1993). The compound produced by strain 9t, which was novel, belongs to the ansamycin family of antibiotic compounds (Carver, 1993). Three actinomycete strains (9t, 9e and 9a) isolated from the Antarctic do not show genotypic divergence in 16S rRNA sequence homology and DNA:DNA hybridization from described species isolated from non-Antarctic climates studies such that the Antarctic strains can be described as a unique indigenous population. However, the production of a novel compound by 9t, a strain of previously described species (*Streptomyces vinaceus* = DSMZ 40257,*Streptomyces streptomicini* = DSMZ 40200), indicates a level of phenotypic variation which makes the

biotechnological potential of actinomycete strains from the Antarctic continent worth further investigation.

Antarctic species "O" and "P" were those most commonly found in the soil samples taken from the Vestfold Hills and Law Base respectively. Of the five Antarctic species found in the present study, species "O" and "P", represented by strains 18a and 19k respectively, showed the least degree of 16S rDNA sequence homology with DSMZ strains. This suggests that novel species, perhaps with novel bioactive compounds, may be selectively enriched by the harsh Antarctic climate.

Gilvocarcin, produced by strain 18a, is strongly active against gram positive bacteria and only weakly active against gram negative bacteria (Nakano *et al.,* 1981). However, 18a and other Antarctic strains belonging to the same species as 18a (species "O"), caused radii of growth inhibition against both *E. coli* and the gram positive test organisms *Staphylococcus aureus* and *Bacillus subtilis* that are of comparable size. One possible explanation for this activity is that species "O" may have produced more than one antibiotic compound. The production of more than one bioactive compound by a single strain of actinomycete is not uncommon (Srinivasan *et al.,* 1991; Champness, *et al.,* 1992; Berwick, 1988). The two strains, 9t and S9b2, members of species "N", both produced antibiotics effective against only one test organism, but each strain produced an antibiotic that was active against a different test organism. Strain 9t produced an antibiotic active against *Staphylococcus aureus,* and strain S9b2 produced an antibiotic active against *Pseudomonas aeruginosa* . The production of two antibiotics by one species (species "O") and production of two different antibiotics by two Antarctic strains which are members of the same species (9t and S9b2, species "N"), highlights the value of further investigation into the biotechnological potential of Antarctic actinomycetes.

4.2 Efficacy of Taxonomic Methods

Fifty two-isolates were obtained from Antarctic soils. The initial task was to sort these isolates into groups of related strains, ideally placing each strain into a group which was comprised of members of the same species, and then to compare these species with described strains. Methods involving phenotypic analysis, chemotaxonomy and molecular taxonomy were used. The efficacy of each of these methods in sorting each of the Antarctic isolates into groups comprised of members of the same species, indicating the

relationships between these species and identifying each species (Table 4.1) is discussed here.

4.2.1 Numerical Analysis of Phenotypic Characters

Results from statistical analysis of phenotypic characters data placed all 52 Antarctic actinomycete isolates into four groups, A, B, C and D (Section 3.5). These results correlated well with the final grouping of isolates based on all taxonomic data (Table 4.1). Groups "B", "C" and "D" determined by numerical analysis of phenotypic characters matched exactly with grouping of strains into species "N", "O" and "P" respectively. Group "A", determined by phenotypic analysis, was divided into species "L" and "N" on the basis of 16S-23SrRNA spacer patterns and 16S rDNA sequence comparison. It is interesting to note that species "L" and "N" which were not differentiated by the numerical taxonomic comparison in the present study were the two Antarctic species most closely related phylogenetically based on comparison of their 16S rDNA sequences.

Colour of aerial spore mass has been considered to be highly reproducible (Williams *et al.*, 1983a), and is consistently expressed in 95% of cases when duplicate cultures of a strain are grown. However, there are many species of streptomycete which are relatively distantly related but which share the same spore colour (Williams *et al.*, 1983a; Kampfer *et al.*, 1991) so in many cases this character could not be used to distinguish between different species isolated from environmental samples. Colour of spore mass was a reliable characteristic in the present study (Table 3.9) for differentiating between species and it was relatively consistent within species. Colour of spore mass did not vary between duplicates of a single isolate but species "L" and "O" usually produced spores sparsely and species "M" did not appear to produce spores at all. Multiple observations were required in order to determine this characteristic for isolates belonging to these three species.

Duplicate cultures of a single strain have been reported to show the same antibiotic effect against a range of eight different test organisms 92.6% (Williams *et al.*, 1983a) of the time. However, in the present study, production or not of antibiotics active against four tests organisms was not consistent for isolates belonging to species "N", "O" and "P" (Table 3.9). Isolates belonging to species "L" and "M" consistently produced no antibiotics effective against any of the four test organisms.

Duplicate cultures of a single strain are reported to produce melanin on Peptone-Yeast Extract-Iron agar 100% of the time (Williams *et al.*, 1983a). The production of melanin on Peptone-Yeast Extract-Iron agar was consistent for all isolates within species except for a single isolate, 22b, which belonged to species "O" (table 3.8). This characteristic distinguished between isolates that belonged to species "L" and isolates that belonged to species "M". However, these two species were not differentiated when all morphological and physiological characteristics were combined in the numerical taxonomic analysis.

Carbohydrate utilization has been reported to be reproducible in duplicates 92.2% of the time using a mean of 25 carbon sources (Williams *et al.*, 1983a). Although carbohydrate utilization patterns were not consistent amongst all isolates of the same Antarctic species, the different carbohydrate utilization patterns of the Antarctic isolates could distinguish between four of the five different species. The four groups that formed in the numerical analysis of all data were able to be distinguished on their utilization pattern of five of the carbon sources: mannitol, trehalose, rhamnose, melibiose and rhamnose, (Table 3.9).

4.2.2 Chemotaxonomy

Members of a number of actinomycete genera can be distinguished on the basis of menaquinone profiles alone (Table 1.7). In all Antarctic isolates the menaquinone profile was the same: 9H6 and 9H8 were the major menaquinones and 9H4 and 9H2 were present as either minor or major components. This is a pattern typical of members of genus *Streptomyces*. In combination with the fatty acid and morphological data the identification of all Antarctic isolates as belonging to genus *Streptomyces* was conclusive.

Once isolates had been placed into groups of related strains on the basis of all taxonomic data it was possible to compare the results of the two methods used for obtaining fatty acid profiles (Table 4.3).

Table 4.3: Comparison fatty acid profiles for Antarctic species L, M, N, O and P, obtained using extraction methods 1 (bold) and 2 (plain text). Values for each fatty acid are the average value for that fatty acid (as a % of the total fatty acids) of all isolates belonging to a single.

Species	Average Fatty Acid Value											
	iso 14:0	iso 15:0	ante-iso 15:0	15:0	iso 16:1	iso 16:0	16:1 w7c	16:0	iso 17:1	ante -iso 17:1	iso 17:0	ante -iso 17:0
L	**1**	**4**	**23**	**1**	**1**	**9**	**12**	**15**	**3**	**4**	**2**	**15**
L	2	4	26	1	2	14	8	8	3	7	2	20
M	**3**	**11**	**17**	**1**	**2**	**15**	**9**	**13**	**5**	**3**	**5**	**10**
M	2	9	19	2	4	18	8	6	6	5	3	12
N	**3**	**4**	**27**	**1**	**2**	**14**	**7**	**9**	**2**	**5**	**2**	**17**
N	6	5	24	1	7	23	4	4	2	5	2	11
O	**2**	**5**	**29**	**2**	**2**	**12**	**11**	**13**	**1**	**4**	**1**	**11**
O	2	10	27	1	5	12	11	6	5	8	2	8
P	**3**	**6**	**27**	**1**	**3**	**18**	**5**	**7**	**3**	**5**	**1**	**13**
P	3	6	30	2	2	18	4	2	4	6	2	15

Fatty acids were extracted from Antarctic isolates by two different methods (Section 2.8.1). In method one non-saponifiable lipids were removed prior to the acidification of the saponified lipid extract. Non-saponifiable lipids were not removed in method two. Comparison of fatty acid profiles obtained using method one the University of Tasmania and method two at the DSMZ showed that there is no value in comparing fatty acid profiles obtained using different methods at different laboratories as a means of identification. A shortcoming of this approach was reported in the literature (Korn-Wendisch and Kutzner, 1991).

Cluster analysis of fatty acid profiles obtained by method one did not result in well defined groups of isolates (Figure 3.3). Isolates belonging to species

"L" (9r, 9c, 9a, S9a, S9c) clustered together, and many isolates within species "O" were closely clustered. However, many isolates were clustered more closely with isolates of other species than with members of their own species. For example, isolate 19c clustered more closely with *Streptomyces griseus* than with any members of its own species. Some isolates from species "N", "O" and "P" were placed into a loose cluster in which members of these three different species were not differentiated. One isolate, 22c, was placed well outside any cluster but other taxonomic data showed that it was a member of species "O".

Cluster analysis of fatty acid profiles obtained by method two resulted in groups of isolates which were better defined than those clusters obtained by comparison of fatty acid profiles obtained using method one (Figure 3.5). Relationships between isolates in cluster analysis of fatty acid profiles from method two also reflected other taxonomic data more closely than did results of cluster analysis from method one. For example, all strains within species "M" and "O" were clustered most closely with members of their own species. However, species ""N" and "P" which could be differentiated by method one could not be differentiated by method two.

The results from method two were more consistent than results from method one with other taxonomic data. Method two was also a faster and simpler method, and therefore less prone to experimental error. Both methods needed to be combined with other taxonomic methods in order to obtain a true picture of the relatedness of isolates.

Fatty acids profiles derived from method two were compared to the DSMZ actinomycete fatty acid database in an attempt to identify them. None of the fatty acid profiles of the Antarctic strains were similar enough to those within the DSMZ database for any of the Antarctic strains to be identified on the basis of this comparison (Table 3.12).

Menaquinone profiles cannot be compared amongst strains in the same manner as fatty acid profiles. Menaquinone proportions change during different growth phases of the microorganism, and it is difficult to regulate growth phase in streptomycetes, which form clumps of hyphae in broth culture. There are also a lesser number of a compounds involved in menaquinone analysis. It is possible to identify and compare variations in up to 20 fatty acids, but only four or five menaquinones were identified, and

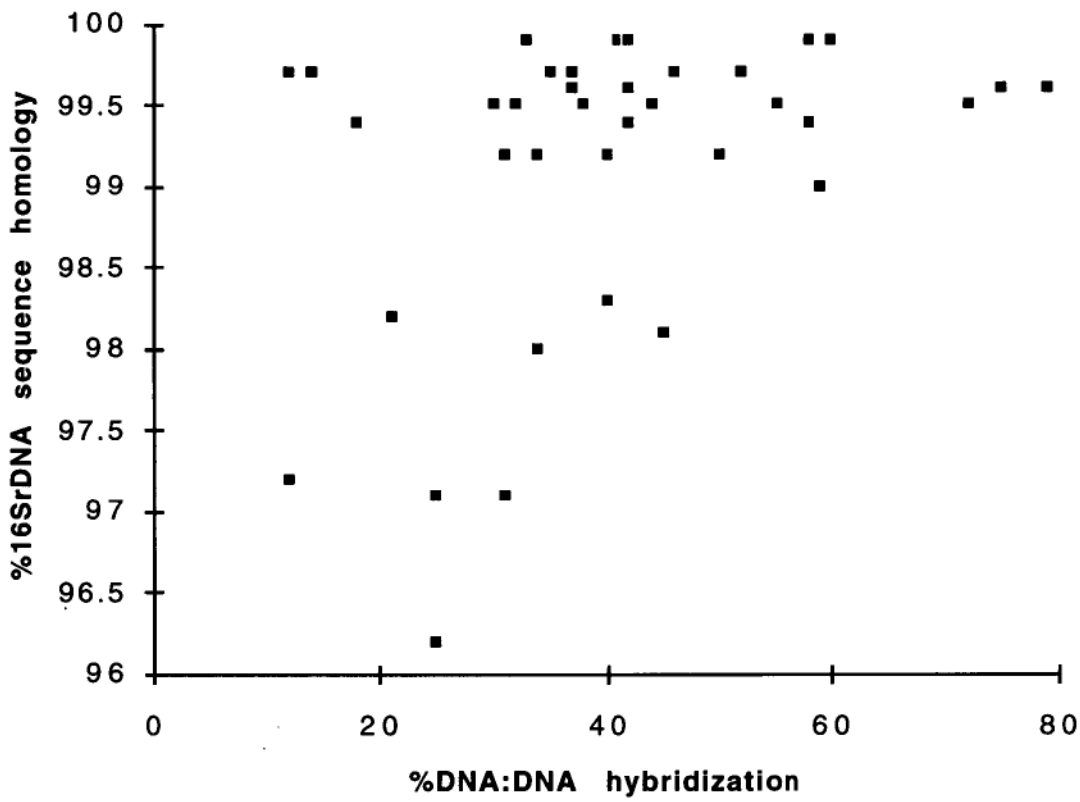
a small variation in the proportion of one of these menaquinones would have a disproportionate influence on the final result.

4.2.3 Molecular taxonomy

The reliability of 16S rRNA comparisons at high levels of similarity to accurately show phylogenetic relationships has been questioned (Fox *et al*, 1992), a constraint supported by the present study. Antarctic strain 9t was 99.9% similar in 16S rRNA sequence to DSMZ strain 40361 (*Streptomyces analatus* type strain) but the strains were 42% similar by DNA:DNA hybridization. In contrast, Antarctic strain 9t showed 99.6% 16S rRNA sequence similarity with DSMZ strains 40200 (*S. streptomycini*) and 40257 (*S. vinaceus*) which were 79% and 75% similar respectively in DNA:DNA hybridization. The inability of 16S rRNA comparisons at these high levels of similarity to accurately identify species may be due to errors in sequencing or because rapid changes in the hyper-variable regions which are given too much weight in the comparison of partial sequences. Compared to phenotypic and chemotaxonomic analysis 16S rDNA sequencing was a relatively expensive and time-consuming method and it was impractical to use it as a method for sorting large numbers of environmental isolates into groups of closely related strains.

There is no linear correlation between 16S rRNA sequence homologies and DNA:DNA hybridization values (Stackebrandt and Liesack, 1993; Stackebrandt and Goebel, 1994). However, Owen and Pritcher (1985) suggested that, in conjunction with other supporting taxonomic data, similarities of between 30 and 65% in DNA:DNA hybridization studies may indicate close relatives. In the present study, a number of DSMZ strains with high 16S rRNA sequences homologies with Antarctic strains also had a high %similarity in DNA:DNA hybridization studies. For example, %hybridization between Antarctic strain 9t and DSMZ strain 40128 was 60%, and sequence 16S rRNA sequences homology between these two strains was 99.9%. The correlation between 16S rRNA sequence homologies and %DNA:DNA hybridization was not linear (Figure 4.1). For example, Antarctic strain 9t had 99.9% sequence homology with DSMZ strain 40361 and 99.4% sequence homology with DSMZ strain 40348 but DNA:DNA hybridization values for 9t and DSMZ strains 40361 and 40348 were 42% and 58% respectively.

Figure 4.1: A comparison of DNA:DNA hybridization values and 16S rDNA homology values. Each point marked on the chart represents a pair of strains compared in the present study by DNA:DNA hybridization. The DNA:DNA hybridization value of each pair is shown on the x axis. The 16S rDNA %sequence homology for each pair is shown on the y axis.



16S rDNA sequence comparison with the DSMZ streptomycete sequence database indicated the problems associated with identification of environmental strains which belong to the genus *Streptomyces* . Comparison of 16S rDNA sequence amongst members of the genus *Streptomyces* indicates that many of the described strains included in this genus are closely related to one another (Rainey and Stackebrandt, 1996, unpublished data). Large numbers of streptomycete strains with a high degree of 16S rDNA sequence homology mean that the relationships between environmental isolates, such as 9a and 18a in the present study, and described species which are represented in the DSMZ 16S rDNA sequence database, can only be accurately determined by DNA:DNA hybridization studies with many strains. It is unreasonable to expect that every investigator will spend their resources on these large DNA:DNA

hybridization studies, especially in industry where interest lies in exploiting actinomycetes for their potential as producers of bioactive molecule rather than in accurate taxonomy. The practical application of the present definition of a species is therefore questionable, at least within the Actinomycetes.

At present, the DSMZ's partial 16S rDNA database for their streptomycete collection has not been released. These sequence results may allow rationalisation of the taxonomy of *Streptomyces*.

A database of Actinomycete relationships based on 16S rRNA sequence comparison and DNA:DNA hybridization studies, along with the spacer pattern or RFLP profile or both for each strain, would aid fast, accurate identification of environmental isolates within a phylogenetically based taxonomy. Extensive DNA:DNA hybridization studies within some actinomycete genera have been conducted (Poschner *et al.*, 1985; Stackebrandt, *et al.*, 1981; Williams *et al.*, 1983a) but no comprehensive study of this nature has been conducted for members of the genus *Streptomyces*.

The five Antarctic species found in the present study were divided into four groups by Amplified ribosomal DNA restriction analysis (ARDRA). Representative strains of each of the five Antarctic species were placed into groups based on ARDRA patterns as follows: (9e and 9e), (9t), (18a) and (19k). ARDRA patterns placed strains 9a and 9e into the same group, which also occurred in the numerical taxonomic analysis. ARDRA was used after isolates had been identified as belonging to particular species and after the partial 16S rRNA sequences for representative strains of each species had been determined. Restriction enzymes which would cleave the 16S rDNA of one Antarctic species but not another at a site at which 16S rDNA sequence varied between the two species were chosen. On the basis of the sequence variation between Antarctic species and the restriction enzymes chosen, it should have been possible to differentiate each of the five Antarctic species on the basis of different DNA fragment banding patterns in gel-electrophoresis following enzyme digestion using four different restriction enzymes. However, the five Antarctic species could only be divided into four groups on the basis of ARDRA results (Table 3.17). ARDRA would not have been the best method to use in placing the 52 Antarctic isolates into groups of related strains. Unless sites of 16S rDNA sequence variation are known prior to enzyme digestion, a large battery of

enzymes would need to used in order to have a reasonable chance of differentiating between strains with a high degree of 16S rDNA sequence homology. Compared to other molecular methods, such as 16S-23S rRNA internal spacer polymorphism and restriction fragment length polymorphism (RFLP), this method is not an efficient and accurate way of sorting environmental isolates into groups of related strains.

The comparison of 16S-23S rRNA spacer patterns was the most accurate way of sorting the Antarctic isolates into groups of closely related strains. Each of the five groups based on 16S-23S rRNA spacer patterns represented a distinct species. However, two unrelated strains can occasionally share the same spacer pattern, and a single species can contain multiple strains. Therefore, groups based on 16S-23S rRNA spacer patterns must be investigated using other taxonomic methods both to confirm that the members of such groups are closely related and to determine the phylogenetic relationships between these groups. In the present study the groupings based on 16S-23S rRNA spacer patterns were supported by a combination of numerical and chemo-taxonomy and 16S rDNA sequence comparison.

4.3 Isolation of Actinomycetes

4.3.1 Environments Favoured by Antarctic Actinomycetes

In this study, actinomycetes were only isolated from soils which were associated with moss or lichen, or human disturbance. A similar result was reported for "molds" found only in Antarctic soils that were associated exclusively with moss and lichen (Meyer *et al.*, 1967).

Actinomycetes were also associated with soil of low electro-conductivity (Tables 3.2 and 3.3). The pH, percentage loss (by weight) of soil following treatment at 600°C, and percentage water by weight of the soils did not correlate with the presence of the actinomycetes. However, the lack of correlation may be due to the small sample size. Previous studies which attempted to relate Antarctic soil conditions to bacterial counts have generally been inconclusive (Parker *et al.*, 1982; Line, 1988), although a correlation occurred between EC and composition of the bacterial population, with higher counts of halotolerant bacteria associated with soils of high EC (Line 1988). The only correlation between actinomycetes and

Antarctic soil conditions was reported by Horowitz (1979) who found that actinomycetes were more common in soils close to a water source.

It is possible that Antarctic species of moss and lichen cannot grow in saline soils and that it is the preference of actinomycetes for the moss and lichen beds which is reflected in these results rather than a preference for soil of low salinity. The low rate of recovery from Antarctic soils of hyphal actinomycetes on seawater compared with that on tapwater 0.01% yeast extract agar does suggest however that the strains recovered in this study were not well adapted to high salt concentrations.

One explanation for the presence of actinomycetes in moss and lichen beds is that they would be provided with a good source of nutrients and a microclimate with a relatively high water activity. However, if nutrients and water availability were the only reasons that high numbers of actinomycetes were found in soils associated with moss and lichen, it would be expected that high numbers of actinomycetes would similarly occur in soils associated with algal mats and algae under translucent rocks, but this was not so. Actinomycetes have not been isolated in high numbers from Antarctic endolithic environments, which are also rich in algal species and have a relatively high level of water availability (Siebert, *et al.*, 1996; Friedmann, 1982). However, it is possible that the algae produce toxic compounds which inhibit the growth and surival of actinomycetes in these locations (Goodfellow and Williams, 1983). The association of mosses and lichens with actinomycetes in the Antarctic environment may be a result of physical trapping of actinomycete spores in moss and lichen beds. Liskens, Bargagli and Focardi (1992) reported pollen grains from over 30 plant genera were trapped in Antarctic mosses. The plants from which the pollen came were distributed throughout all circumpolar continents indicating that prevailing winds are capable of carrying microscopic particles, including microorganisms and their spores, to the Antarctic. It is possible that some actinomycete spores germinate and grow once in the moss and lichen beds.

Supporting evidence for actinomycete growth in the moss and lichen was the presence of actinomycete propagules in soil near to such moss and lichen beds (soil nine). The recovery of actinomycetes from soil nine would suggest that the actinomycetes within the nearby moss and lichen bed were producing spores which were blown or washed out. Further evidence is that the same strains were isolated from a number of sites, 9, 17, 18 and 22 and that species "O" dominated the actinomycete count at most of these

sites. If the actinomycete isolates originated only from lyophilized wind-borne spores, a wider range of strains would be expected and the population would not be dominated by a few taxonomic types.

There was no obvious correlation between numbers of other microcolony-forming bacteria in antarctic soils and numbers of actinomycetes, although there may have been some correlation with the species composition of microbial populations and numbers of actinomycetes. For example, a large coccoid bacterium that formed tetrads (tentatively identified as a *Micrococcus sp.*) was very common in ornithogenic soils but did not occur in samples from which hyphal actinomycetes were isolated.

4.3.2 Methods of Isolation

Micromanipulation

Most actinomycetes which produce antibiotics are capable of forming hyphae at some stage of their life cycle (Table 1.4). It was therefore decided to selectively isolate hyphal forming actinomycetes. It was also considered that this method would give an indication of relative abundance of actinomycetes compared to other microorganisms, which would not have been possible if more selective media had been used. A disadvantage of micromanipulation is that it does not allow enrichment of those more rare and therefore perhaps more interesting non-hyphal actinomycetes, and it may miss those strains in which the hyphal stage is brief. However the present study was a general survey of actinomycetes in Antarctic soils rather than an attempt to isolate rare forms, so it was more useful to isolate as wide a range as possible of the most common types. Micromanipulation was also used as one aim of this study was to determine whether there was a unique indigenous actinomycete population in Antarctic soils, which would be easier to determine through comparison of the more common soil isolates with known strains from temperate regions. If many new and divergent strains of actinomycete were isolated one could speculate that a unique actinomycete microbiota was endemic to Antarctica.

Micromanipulation also allows isolation of microorganisms with a distinctive morphology, without the need for pre-treatments of samples or special media to select for the desired organism. The disadvantage of pre-treatments and selective media is that they select against particular un-desired organisms but it is impossible to select for unknown organisms

which have unknown growth preferences. A low nutrient media can be used with micromanipulation which will allow a wide range of microorganisms to grow for a number of weeks without the problem of plates becoming overgrown with un-wanted microorganisms. Most colonies remained as microcolonies during isolation. A similar result might be achieved with large numbers of dilution plates but this requires some modification of the media to ensure fungi and gliding bacteria do not take over the plates and very large numbers of plates would be required to survey the same number of colonies. Dilution plating can be used to target specific "rare" actinomycete genera effectively and this would be necessary before drawing any conclusions about the presence of such genera in Antarctic soil.

Heat

It was considered that heating the soil would both remove unwanted organisms (Labeda and Shearer, 1990) and aid in spore germination (Hirsch and Ensign, 1976b). However, heat treatment resulted in reduced numbers of actinomycete microcolonies from all soils. It is possible that the five species isolated from Antarctic soils had some physiological adaptations to the low temperatures in the Antarctic environment and these adaptations resulted in these strains being unable to survive the heat treatment. This provides some support for the theory that these bacteria were indigenous to the Antarctic.

The strains which were isolated from heat treated soils were 19j, 19k and 22c. It is interesting to note that isolates belonging to species "O", which dominated untreated samples, were not recovered from heat treated soils. There was no significant difference in proportion of saturated fatty acids between any of the Antarctic species.

Salinity

The use of sea-water based media reduced numbers of actinomycetes recovered from soils, but that reduction was largely of species "O" and "P". This result, combined with the results of soil examination which showed no actinomycetes isolated from soils with high EC, suggests that species "O" and "P" were not halotolerant. Comparable numbers of species "L", "M" and "N" were obtained on salt water and tap water media. Species "L", "M" and "N" were halotolerant to the extent that they were isolated in equal

numbers on tap water and salt water based agar. Kutzner (1991) reported that *S. analatus* (in which Antarctic species "N" can be accommodated) can grow in the presence of 10%(w/v) NaCl.

Conclusions

1. The fifty two hyphal actinomycetes isolated from Antarctic soil were members of five different species, nominated "L", "M", "N", "O" and "P". Each of these species could be accommodated within the genus *Streptomyces*. Two of the Antarctic species could be accommodated within described species; these were Antarctic species "M", identified as *S. analatus* (DSMZ strain 40361), and Antarctic species "N", identified as *S. vinaceus* (DSMZ strain 40257). Antarctic species "O" and "L" were not shown to belong to any described species but they were not compared by DNA:DNA hybridization studies to every species with which they showed 97% or greater 16S rDNA sequence homology when compared to the DSMZ streptomycete sequence database. Therefore they cannot be described as new species given the current convention of only defining species on the basis of DNA:DNA hybridization. Antarctic species "P" was compared by DNA:DNA hybridization studies to every species with which it showed 97% or greater 16S rDNA sequence homology when compared to the DSMZ streptomycete sequence database and therefore it can be described as a new species.

2. The four Antarctic species "L", "M", "N", and "O" were found only in soils associated with moss and lichen in the Vestfold Hills. Species "O" was isolated from more than one soil sample and in the greatest numbers. Species "L", "M", "N", and "O" represent the dominant actinomycete microbiota in soils from the Vestfold Hills. The evidence suggests that these species are capable of growth during part of the Antarctic year. Antarctic species "P" was found in one soil in an area heavily used by humans and their vehicles. The origin and ability of species "P" to grow in the Antarctic soil from which it was isolated is uncertain. Novel actinomycete species can be isolated from Antarctic soils, and these species may be capable of growth in Antarctic soils associated with moss and lichen but the Antarctic actinomycete microbiota does not consist of organisms unique to this environment.

3. Antarctic strain 18a produced the antibiotic compound gilvocarcin and Antarctic strain 9t produced an antibiotic compound, that belonged to the ansamycin family of antibiotics, but was a novel structure. The production of a previously un-described antibiotic compound by strain 9t indicates that although actinomycetes are found in relatively low numbers in Antarctic soils they do have potential for biotechnological exploitation.

4. A combination of 16S-23S rRNA spacer patterns, phenotypic analysis, and fatty acid comparisons clarified the grouping of Antarctic strains in the present study. Although 16S-23S rRNA spacer patterns accurately grouped isolates into single species, the groups determined by 16S-23S rRNA spacer pattern comparison required further investigation and confirmation by other taxonomic methods. Neither phenotypic analysis nor chemotaxonomy were alone able to place all isolates into groups consisting of a single species, but of these two methods phenotypic analysis placed the isolates into more well defined clusters of isolates belonging to the same species. 16S rDNA sequence comparison amongst Antarctic species and between Antarctic strains and the 16S rDNA sequences in the DSMZ streptomycete database gave some indication of the relationships between strains but it was necessary in most cases to clarify these relationships by DNA:DNA hybridization studies. The number of DNA:DNA hybridizations required in order to clarify all relationships was impracticably large and indicated the need for rationalising the taxonomy of genus *Streptomyces*. These results also indicated that there is a need for redefining the criteria for species definition if it is to have practical application.

5. Micromanipulation was a useful method for isolating members of the genus *Streptomyces* from Antarctic soil. In order to determine whether other actinomycete genera are present in Antarctic soils methods which targeted specific genera would need to be employed.

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Appendix 1: Reagents and Media

A.1.1 Solutions for Determining Soil Characteristics

Saturated Calcium Sulphate Solution

Add 7 g calcium sulphate (dihydrate) to 2 L of distilled water
Shake for 2 hours at 20°C
Filter through Whatmann No. 2 filter paper
Store at 20°C

0.02M Potassium Chloride

Dry KCl at 102°C for 1 hour, then allow to cool in a desiccator
Dissolve 1.492g in one litre of water
Store at 20°C

A.1.2 Reagents for Fatty Acid Extraction: Method 1

Saponification Reagent

NaOH	45 g
Methanol	150 mL
Distilled water	150 mL

Extraction Solvent

Hexane	80 mL
Methyl-t-butyl ether	10 mL

Acidifying Agent

H_2SO_4	20 mg
Distilled water	100 mL

Methylation Reagent

Chloroform	1 mL
HCl	1 mL
Methanol	10 mL

2M Sulphuric Acid

H_2SO_4	58.8 g
Distilled water	300 mL

A.1.3 Reagents for Fatty Acid Extraction: Method 2

Saponification Reagent

NaOH	45 g
Methanol	150 mL
Distilled water	150 mL

Add water and methanol to NaOH pellets in bottle.
Stir until pellets have dissolved.

Extraction Solvent

Hexane	200 mL
Methyl-t-butyl ether	200 mL

Methylation Reagent

6.00N HCl	325 mL
Methanol	275 mL

Add acid to methanol while stirring

Base Wash

NaOH	10.8 g
Distilled water	900 mL

Add water to pellets in bottle.
Stir until pellets are dissolved.

A.1.4 Reagents for Molecular Taxonomy

Saline-EDTA

NaCl	2.19 g
EDTA	0.93 g
Distilled water	250 mL

Binding Buffer

Tris-HCl	3.94 g
Na_2-EDTA	1.86 g
$NaClO_4$	421.32 g
Distilled water	500 mL

Adjust to pH 7.5
Autoclave

Wash Buffer

Tris-HCl	1.576 g
Na_2-EDTA	0.372 g
NaCl	22.177 g
Distilled water	500 mL

Adjust to pH 7.6
Autoclave and add equal volume of 100% Ethanol

Urea Buffer

Urea	240.24 g
$Na_2HPO_4.2H_2O$	10.68 g
$NaH_2PO4.H_2O$	8.28 g
Water	500 mL

Adjust to pH 6.8

Phosphate Buffer 1

$Na_2HPO_4.2H_2O$	0.62 g
$NaH_2PO4.H_2O$	0.48 g
Distilled water	500 mL

Adjust to pH 6.8

Phosphate Buffer 2

$Na_2HPO_4.2H_2O$	17.8 g
$NaH_2PO4.H_2O$	13.8 g
Distilled water	500 mL

Adjust to pH 6.8
or

Phosphate Buffer 1	35 mL
Distilled water	965 mL

Adjust to pH 6.8

20 X Standard saline citrate (SSC)

NaCl	8.6 g
Na-citrate	4.41 g
Distilled water	1000 mL

5mM EDTA-Na_2

EDTA-Na_2 (Sigma)	1.86 g
Distilled water	1000 mL

10X TBE

Tris	107.8 g
Boric Acid	55 g
EDTA (Sigma)	7.4 g

Gel Loading Dye

Glycerin	300 mL
Bromophenol blue	5 mg
Water	700 mL

Polyacrylamide solution

Acrylamide	38 g
Bis-Acrylamide	2 g
Distilled water	1000 mL

A.1.5 Buffers for ARDRA's

New England Biolabs NEBuffer 1

Bis Tris Propane-HCl	10 mmol/l
$MgCl_2$	10 mmol/l
Dithioethrit	1 mmol/l

pH 7.0 at 25°C

New England Biolabs NEBuffer 2

Tris-HCl	10 mmol/l
NaCl	50 mmol/l
$MgCl_2$	10 mmol/l
Dithioethrit	1mmol/l

pH 7.9 at 25°C

New England Biolabs NEBuffer 3

Tris-HCl	50 mmol/l
NaCl	100 mmol/l
$MgCl_2$	10 mmol/l
Dithioethrit	1 mmol/l

pH 7.9 at 25°C

Boehringer Mannheim, SuRe/Cut Incubation Buffer M 2

Tris-HCl	10 mmol/l
NaCl	50 mmol/l
$MgCl_2$	10 mmol/l
Dithioethrit	1 mmol/l

pH 7.5 at 37°C

A.1.6 Growth Media

Salt solutions for carbohydrate growth media

1. High salt solution:

KH_2PO_4	2.38 g
$K_2HPO_4.3H_2O$	5.56 g
Agar	1.5 g
Distilled water	100 mL

pH: 6.8

2. Low salt solution:

$(NH_4)_2SO_4$	2.64 g
$MgSO_4.7H_2O$	1 g
$CuSO_4.5H_2O$	6.4 mg
$FeSO_4.7H_2O$	1.1 mg
$MnCl_2.4H_2O$	7.9 mg
$ZnSO_4.7H_2O$	1.5 mg
Agar	1.5 g
Distilled water	100 mL

pH: 6.8

Peptone-Yeast Extract-Iron Agar

Glycerol	15 g
L-arginine	5 g
L-tyrosine	1.0 g
L-methionine	0.3 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
$CuSO_4.5H_2O$	0.01 g
$CaCl_2.2H_2O$	0.01 g
$FeSO_4.7H_2O$	0.01 g
$ZnSO_4.7H_2O$	0.01 g
$MnSO_4.4H_2O$	0.04 g
Agar	15 g
Distilled water	1000 mL

DSM-65 Streptomyces Agar

Glucose	4 g
Yeast extract	4 g
Malt extract	10 g
$CaCO_3$	2 g
Agar	12 g
Distilled Water	1000 mL

pH: 7.2

DSM-65 Streptomyces Broth

Glucose	4 g
Yeast extract	4 g
Malt extract	10 g
Distilled Water	1000 mL

pH:7.2

Micromanipulation Agar

Yeast Extract	0.01 g
Agar	15 g
Distilled water	1000 mL

Seawater Micromanipulation Agar

Yeast Extract	0.01 g
Agar	15 g
Filtered Seawater	1000 mL

Sporulation Agar (DSM)

Yeast Extract	1 g
Beef Extract	1 g
Tryptose	2 g
$FeSO_4$	10 ppm
Glucose	10 g
Agar	15 g
Distilled water	1000 mL

Adjust to pH 7.2

Modified Sporulation Agar

Yeast Extract	1 g
Beef Extract	1 g
Tryptose	2 g
$FeSO_4$	10 ppm
Glucose	3 g
Agar	15 g
Distilled water	1000 mL

Adjust to pH 7.2

Sporulation Broth

Yeast Extract	1 g
Beef Extract	1 g
Tryptose	2 g
$FeSO_4$	10 ppm
Glucose	3 g
Distilled water	1000 mL

Adjust to pH 7.2

Trypticase Soy Yeast Agar

Trypticase Soy Broth	5 g
Yeast Extract	1 g
Agar	15 g
Distilled water	1000 mL

Adjust to pH 7.0

Trypticase Soy Broth

Casein Digest	15 g
Soy Extract	5 g
NaCl	5 g
Distilled water	1000 mL

Adjust to pH 7.4

Glucose-Yeast-Malt extract (GYM) Agar

Glucose	4 g
Yeast Extract	4 g
Malt Extract	10 g
$CaCO_3$	2 g
Agar	12 g
Distilled water	1000 mL

Adjust to pH 7.2

Glucose-Yeast-Malt extract (GYM) Broth

Glucose	4 g
Yeast Extract	4 g
Malt Extract	10 g
Distilled water	1000 mL

Adjust to pH 7.2

Inorganic Salts-Starch Agar

Soluble starch	10 g
$(NH_4)_2SO_4$	2 g
K_2HPO_4(anhydrous)	1 g
$MgSO_4.7H_2O$	1 g
NaCl	1 g
$CaCO_3$	2 g
Trace salts	1 mL
Agar	12 g
Distilled water	1000 mL

Trace Salts:

$FeSO_4.7H_2O$	0.1 g
$MnCl_2.4H_2O$	0.1 g
$ZnSO_4.7H_2O$	0.1 g
Distilled water	100 mL

Appendix 2: Identification of Menaquinones

A survey of the literature was used to construct a table (A.2.1) of microorganisms which produced a wide variety of menaquinones. From this table five microorganisms were chosen which would cover the full range of menaquinones likely to be produced by actinomycetes from reading of the literature. The five species used were *Corynebacterium flaccumifaciens* (ATCC 33802), *Arthrobacter globiformis* (ATCC 8010), *Streptomyces griseus* (DSM 40236), *Staphylococcus aureus* (ATCC 660) and *Flavobacterium sp.* (ACAM 78, NCMB 259).

A series of standard curves were determined on semi-log paper (Tamaoka *et al.*, 1983; Tamaoka, 1986) using standardised elution times of menaquinone peaks (Fig. A.2.1, Table A.2.2). Peaks were standardised by running a sample of *Streptomyces griseus* menaquinones immediately before and after the menaquinones for each of the other four standard organisms. Elution time for MK9(H_8) was given a value of 100 and the elution times for other peaks were standardised using the formula:

$$\frac{100}{(9(H_8)_1+9(H_8)_2)\div 2} \times MK$$

where:

$9(H_8)_1$ = elution time for MK9(H_8) in first run of *Streptomyces griseus*
$9(H_8)_2$ = elution time for MK9(H_8) in second run of *Streptomyces griseus*
MK = elution time for standard or test menaquinones.

This standardising formula was applied to each of the menaquinones extracted from the test organisms. Standardisation was performed in order to take into account drifts in elution time caused by slight variation in temperature and pressure of the column, changes in the batch of solvent used and changes in the column packing over time.

Table A.2.1: Menaquinones profiles for a range of Gram positive bacteria. 1=minor or trace, 2=minor, 3=major. This table was constructed from a number of references in order to choose as standards for menaquinone analysis of actinomycetes a range of organisms which would cover the entire possible range.

STRAIN	Ref.	ATCC number	Menaquinones Present																										
			6	6/2	6/4	7	7/2	7/4	7/6	7/8	8	8/2	8/4	8/6	8/8	9	9/2	9/4	9/6	9/8	9/10	10	10/2	10/4	10/6	10/8	11	12	13
Arthrobacter atrocyaneus, ramosus, polychromogenes and *citreus*	11	-------					2					2				2	3												
Arthrobacter globiformis AJ1422	12	-------		1			1					2					3						1						
Arthrobacter globiformis *	10	8010					2					2				2	3												
Brevibacterium stationis *	10	14403					2				2	3				2	3												
Brevibacterium lyticum	11	-------						2				2				2	3												
Microbacterium lacticum and*liquifaciens*	11	-------									2					2						2				3	3		
Brevibacterium imperiale (nee. *Microbacterium imperiale*)	11	8365									2					2						2				3	3	2	
Corynebacterium bovis and *callunae*	10	-------					2					2				2	3												
Corynebacterium flaccumfaciens	3, 10	33802	1			1					3					3						1							
Corynebacterium aquaticum *	9, 10	14665				2					2					2						3				3	2		
Corynebacterium mediolanum	9	14004				2					2					2						2				3	3	1	
Corynebacterium flavidum C35 *	2	6940				1						3	2				1												
Corynebacterium equi C7 *	2	6939					2				2	3	2				2												
*Nocardia brasiliensis**	2	19296			1			2				2	3																
Nocardia caviae	2	14629			1			2				2	3																
Nocardia vaccinii	2	11092			1			2				2	3																
Nocardia asteroides	4	19247			1			2				2	3																
Nocardia farcinica (NCTC 4524)	2	-----										1	1			1	3	1											
Micropolyspora brevicatena (Syn. *Nocardia brevicatena*)	2	15725			2			2				2	3																

Micropolyspora faeni * (nee *Saccharopolyspora rectivirgula*)	2	15347								1	1	1		1	3	2	2				1	1
Actinoplanes caeruleus LA176	5	33937			1		1				1	1		1	3	2						
Dactylosporangium auranticum *	5	23491				1	1			1	1	1		1	1	2	3					
Micromonospora purpureochromogenes D18	5	27007												2	2	2	2				2	3
Micromonospora parva	5	27358			1	1			1					1	1	1				1	3	2
Micromonospora melanospora (DSM 43126, LBG A311,IVP 583)	5	-------				1				1	1				1	1				1	3	2
Actinomadura pelletieri A19	4	-------									2	2		2	2	2	3	2				
Actinomadura citrae A138 *	6	27887											1	1	2	3	2	1				
Actinomadura coerulea A226 (INA 765) *	6	33576								1	1			1	1	2	3	1				
Actinomadura helvata A5 *	6	27295								1	1			1	1	3	2					
Actinomadura malachitica A139 *	6	27888											1	1	2	3	2	1				
Actinomadura dassonvillei A14 *	2	23218									1			1	1	1				2	3	2
Actinomadura dassonvillei A15	2	23219							1	1	1			1	1	1			1	2	3	2
Actinomadura dassonvillei A114	2	------								1			1	1	1	1			1	2	3	2
Microtetraspora niveoalba G103	6	27301							1	1			1	1	3	2	1					
Microellobosporia violacea M297 *	4	-----							1	1	1			1	2	3	2					
Streptomyces somaliensis (pasteur 395)	2	-------			1	1	1				1			1	1	2	3					
Streptomyces somaliensis (pasteur 314)	2	-------			1	1	1				1			1	1	2	3	1				
Streptomyces catenulae(ISP 5258) *	4	12476							1	1	1	1	1	1	2	3	1					
Streptomyces cyaneus nee:*coerulescens* (NCB9616)	1	19896													2	3	3					
Streptomyces griseus	13	DSM 40236									1			1	2	3	2					
Streptomyces cyaneogriseus (ISP 5534)	4	27426							1	1	1			1	2	3	2					
Streptovertocillium cinnamonium (ISP5005) *	4	11874								1	1	1		1	2	3	2					
Streptovertocillium netropsis(baldacii) (ISP5259) *	4	23940								1	1	1		1	2	3	1					
Lactobacillus mali *	8	27053		2				3					3						2			
Bacillus pasteuri (NCTC 4822) *	8	11859	2	3																		
Bacillus thuringiensis	11	-----		3				3														
Bacillus subtilis	11	-----		3																		

Bacillus pumilus (NIAH1021)	3	-----	2	3		1	
Streptococcus lactis	11	-----	1	1		1	3
Staphylococcus aureus	11	-----		1		3	1
Staphylococcus epidermis	11	-----	1	3		1	
Vibrio costicola (NCMB 701) *	7	33508	2	2	3		

* type strain

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8 Collins, M. D., Shah, H. N., Minnikin, D. E. (1980)
9 Collins, M. D. and Jones, D. (1980)
10 Collins, M. D. and Goodfellow (1979)
11 Collins, M. D. and Jones, D. (1981)
12 Tamaoka, J., Katayama-Fujimara, Y. and Kuraishi, H. (1983)
13 Kroppenstedt, (1985)

Table A.2.2. Elution times of menaquinones from standard organisms. These elution times were used to construct a standard curve which was used for identification of menaquinones extracted from Antarctic actinomycetes.

Species	Elution times (minutes)	Converted elution times[1]	Peak identity (MK)[2]
Streptomyces griseus	12.90	100	9(H)8
	10.98	85.12	9(H)6
	9.28	71.94	9(H)4
	8.06	62.48	8(H)6
	7.62	59.07	9(H)2
Staphylococcus aureus	6.78	52.58	9
	4.800	37.21	8
	4.150	32.17	7(H)2
	3.320	25.74	7
Corynebacterium flaccumfaciens	6.77	52.46	9
	4.81	37.27	8
	4.05	31.43	7(H)2
	3.45	26.77	7
Flavobacterium sp.	2.59	19.27	6
Arthrobacter globiformis	7.842	60.78	9(H)2
	5.50	42.6	8(H)2

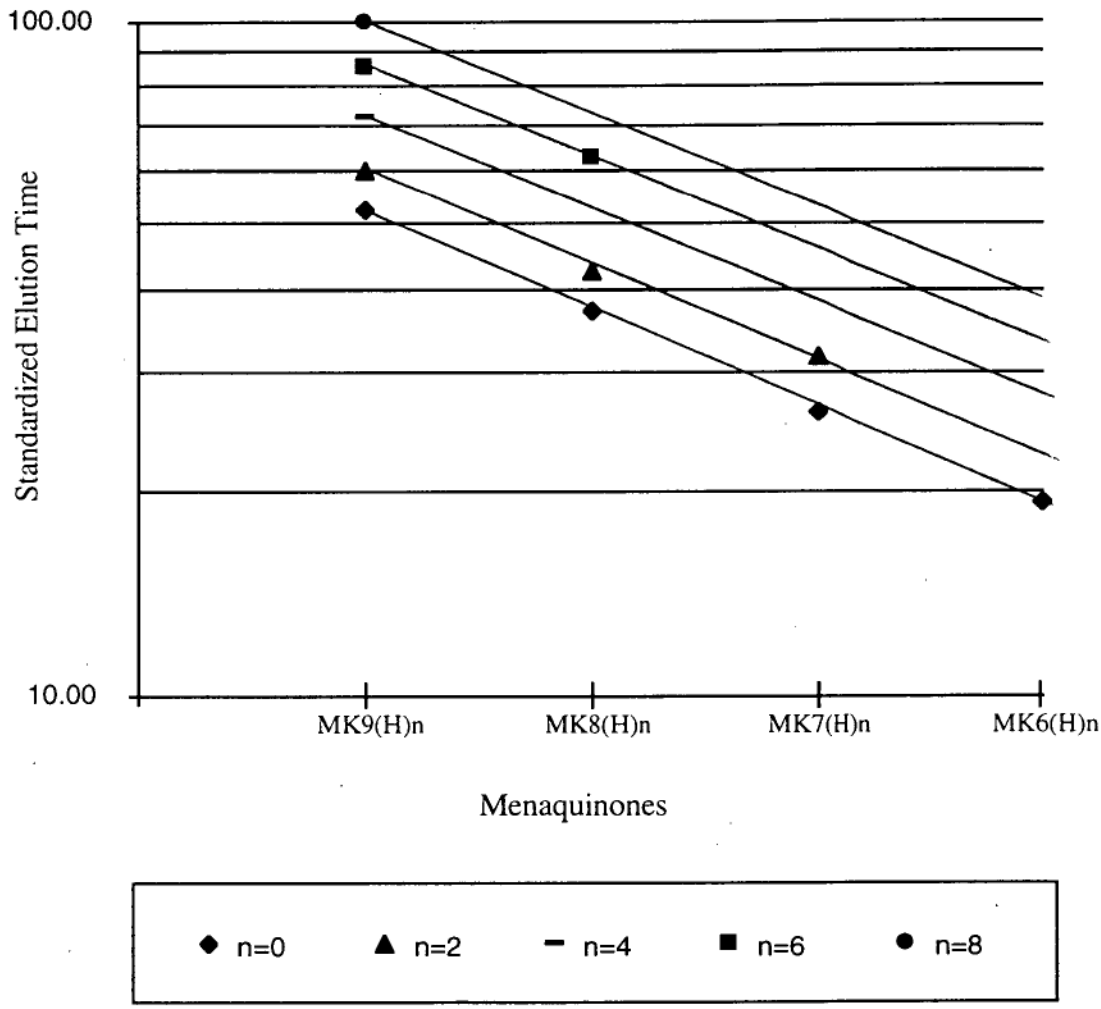
[1] Converted elution times were calculated using the fromula as shown above in appendix 2.

[2] Menaquinone peaks from standard organisms were identified through comparison with table A.2.1 which shows menaquinones reported in the literature for each of the species used as standards.

Table A2.3: Standardised HPLC elution times of menaquinones extracted from from standard organisms.

Menaquinone	Average standardised elution times
9h8	100
9h6	85.12
9h4	71.94
9h2	59.93
9	52.52
8h6	62.48
8h2	42.6
8	37.24
7h2	31.8
7	26.26
6	19.27

Figure A.2.1: Standard curves for identification of menaquinones, plotted on a semi-logarithmic scale. The points on the standard curves were determined by extraction and analysis of menaquinones from microorganisms with known menaquinone profiles (Table A.2.1). Standardized elution times were determined as shown in Table A.2.3. Construction of curves was based on the method of Tamoaka, 1986.



Appendix 3: Enzyme Cleavage Sites for Restriction Enzymes

Table A.3.1: A range of restriction enzymes were investigated. The site each enzyme cleaved was compared to the partial 16S rRNA sequences for five Antarctic isolates: 9a, 9e, 9t, 18a, and 19k. The number of fragments resulting from a digestion, the size of the fragments, and the ability of an enzyme to differentiate between isolates was considered. Ideally the enzyme should cleave two different strains at different sites and the fragments should vary in size such that they might be be easily distinguished by gel electrophoresis. Information for enzyme cleavage sites was taken from the website http://www.medkem.gu.se/cutter/. Enzymes used in the present study are underlined.

Enzyme	Cleavage site	No. of cleaveage sites for majority of isolates*	Expected grouping of five Antarctic strains
Hae III	GGCC	11	(9a, 9e, 9t, 18a) (19k)
Tru 91	TTAA	4	(9a, 9e, 9t, 19k) (18a)
Hinf 1	GANTC	3	No differentiation
Sau 3A	GATC	1	No differentiation
HPA II	CCGG	10	(9a, 9e, 9t) (18a) (19k)
Nci I	CC(G/C)GG	3	No differentiation
NSP 1	(A/G)CATG(T/C)	2	(9a, 9e, 9t, 18a) (19k)
Bmy I	G(G/A/T)GC(C/T/A)C	3	(9a, 9e, 9t) (18a) (19k)
Cvi RI	TGCA	6	(9a,18a) (9e, 9t, 19k)
Aci I	CCGC	12	(9a, 9e) (9t) (18a, 19k)
Cfo I and Hin Pi	GCGC	3	(9a, 9e) (9t, 18a) (19k)
Nla III	CATG	6	(9a, , 9e18a) (9t) (19k)
Tsp EI	AATT	2	No differentiation
Nde II	GATC	1	No differentiation
Alu I	AGCT	5	No differentiation
Mvn I	CGCG	4	(9a, 9e, 9t) (18a, 19k)
Mae II	ACGT	5	No differentiation
Taq I	TCGA	3	No differentiation
Rsa I	GTAC	4	(9a, 9e, 9t, 18a) (19k)
Tfi I	GA(A/T)TC	1	No differentiation
GvbS I	GC(A/T)GC	7	No differentiation
Nci I	CC(G/C)GG	7	No differentiation
Tsp 45 I	GT(G/C)AC	3	(9a, 9t, 18a, 19k) (9e)
Cac 8 I	GCNNGC	6	(9a, 9e, 9t) (18a) (19k)
Dsa V and Scr fI	CCNGG	5	(9a, 9e, 9t, 18a) (19k)
Mae III	GTNAC	3	(9a, 9t, 18a, 19k) (9e)

* This number was determined by comparing enzyme cleavage site to partial 16S rDNA sequences. It is possible that a greater number of cleavages could occur through enzyme action on cleavage sites which were not counted as not all sites had been sequenced.

Appendix 4: Freeze Drying

From C. Nichols (unpublished method, 1994)

4.1 Preparation of Ampoules

Strains were freeze dried and stored in the Australian Collection of Antarctic Microbes (ACAM) for long term preservation. Each strain was assigned an ACAM number. Six labels with their assigned ACAM number and date of freeze drying were prepared for each of the strains on a laser printer. The labels were cut with a blank space of approximately 10mm to the left of the number and date, and to a width enabling them to fit in the freeze drying ampoules. The labels were placed in the ampoules with the number and date at the top. 0.5mL of a 10% skim milk solution was added to each of the tubes, pipetted directly into the bottom of the ampoule without wetting the top of the label. The ampoule was plugged with a 2.5cm cotton wool swab wrapped in sufficient cotton wool to give a snug fit. The ampoules were placed in a beaker which was covered in aluminium foil and sterilised in an autoclave for 30 minutes at 105°C, then allowed to cool to room temperature before being placed in a -20°C freezer and left until the milk solution was frozen. When the milk was frozen the beaker was placed in a freeze drier which was run overnight. The beaker was then removed for storage at -20°C until the ampoules were needed.

4.2 Freeze Drying of Cultures

Isolates were grown on Sporulation Agar (Appendix 1). The beaker containing sterile ampoules was removed from the refrigerator and allowed to come to room temperature. A contamination free plate in an exponential stage of growth was selected and the following procedure was conducted in the laminar flow hood. Sterile forceps were used to pull the cotton plug from the sterile ampoule. Ten drops of sterile sporulation broth (see appendix 1) were dropped onto the agar plate with a sterile pasteur pipette. A suspension of cells and spores was made by scraping the plate with a sterile strip of metal. One drop of the suspension was transferred into the ampoule, and the cotton swab was placed back in the top of the ampoule and then pushed down such that the top of the swab was 2 cm below the top of the ampoule. The transfer of a drop of suspension to a sterile pre-prepared ampoule was repeated six times for

each strain. The ampoule was then connected to the freeze drier and left to run for 4 to 5 hours. The ampoules were sealed by melting the ends closed with a high temperature gas flame while still attached to the freeze drier so that the interior of each ampoule was still a vacuum. Two ampoules, chosen at random from a batch, were broken open and checked to ensure that the cells could be revived and were not contaminated. The remaining ampoules were stored at 4°C.